SOME SEROLOGICAL STUDIES IN THE HORSE IN RELATION TO THE PATHOGENESIS OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE.

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I declare that the contents of this thesis are my own work and have not been presented to any University other than the University of Edinburgh.

June 1981.

I dedicate this work to my wife Joy and to my family.

Chan eil eòlas, chan eil eòlas
air crìch dheireannaich gach tòrachd
no air seòltachd nan lùban
leis an caill i a cùrsa.

There is no knowledge, no knowledge,
of the final end of each pursuit,
nor of the subtlety of the bends
with which it loses its course.

From: "Coilltean Ratharsair"
"The Woods of Raasay"
Sorley MacLean.

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SUMMARY

The study described in this thesis was designed to examine some serological factors which may be involved in the pathogenesis of chronic obstructive pulmonary disease (C.O.P.D.) of horses.

In the first section, zone electrophoresis of normal horse serum on agarose gels (pH 8.6) was studied and the serum electrophoretic profiles of normal and C.O.P.D. affected horses and ponies were compared. No differences between the serum electrophoretic profiles of healthy and C.O.P.D. affected horses and ponies were observed which could be attributed to the presence of the disease.

In the second section, the nature of the two major, electrophoretically distinct antiproteases in horse serum was investigated prior to examining the possible association of antiprotease deficiency with the onset of C.O.P.D. in the horse, analogous to the association of the inherited dysproteinaemia of alpha-1 antitrypsin deficiency and chronic lung disease in man. The electrophoretically faster antiprotease, a functional homologue of human alpha-1 antitrypsin, was shown to appear in the prealbumin region of horse serum after acidic starch gel electrophoresis (pH 4.3). This polymorphic antiprotease corresponded to the allele products of the Pr locus of horse serum described by Braend (1970). The genetically determined polymorphism of the Pr antiprotease was examined by acid starch

gel electrophoresis, isoelectric focusing and immunofixation electrophoresis. The occurrence of a second antiprotease in the acidic prealbumin region of horse serum was postulated, although its nature remains to be established.

The electrophoretically slower antiprotease of horse serum was identified as alpha-2 macroglobulin, and was shown to contribute 48 percent of the total serum antiproteolytic activity. As in man, horse alpha-2 macroglobulin is able to inhibit the proteolytic activity of trypsin, but has only limited inhibitory activity on its esterolytic activity. Native alpha-2 macroglobulin was shown to possess esterase activity and the possible association of the macroglobulin and plasma pseudocholinesterase is discussed. No inherited polymorphism of horse alpha-2 macroglobulin was observed.

The Pr antiprotease allele frequencies in healthy and C.O.P.D. affected Thoroughbred horses were compared and no significant differences were observed. There was however an apparently increased frequency of the PrW allele amongst C.O.P.D. affected horses and ponies of mixed breeding, although the significance of this observation could not be established. Significantly increased levels of immunochemically measured circulating Pr protein were observed in a C.O.P.D. affected population, although no corresponding increase in biochemically measured serum trypsin inhibitory capacity (STIC) was observed in this same population.

It was concluded that serum antiprotease deficiency and consequent predisposition to the development of C.O.P.D. was unlikely to occur in the horse, although a possible deficiency of local bronchiolar anti-proteases, resulting in an increased chance of hyper-sensitization to the protease antigens of the fungi commonly incriminated in C.O.P.D., could not be excluded.

In the third section the occurrence of a serum homocytotropic antibody in the horse, homologous to human IgE, was investigated. A passively transferable homocytotropic antibody against Culicoides pulicaris was demonstrated in the serum of horses and ponies affected with recurrent seasonal dermatitis. Like human IgE, this antibody is heat-labile, susceptible to thiol reducing agents and persists for long periods in homologous skin. The elution characteristics of the horse antibody on DEAE-anion exchange chromato-graphy are similar to those of human IgE. Anti-human IgE was shown to induce reversed anaphylaxis-like reactions in horse skin and immunfluorescent studies provided preliminary evidence of the binding of anti-human IgE to horse mast cells. These observations on the equine homocytotropic antibody satisfy Vaerman's (1970) criteria of interspecies protein homology suggesting that the antibody is homologue of human IgE.

CHAPTER I

GENERAL INTRODUCTION

1. The object of the experimental work described in this thesis is to investigate some serological and immunological factors which may be involved in the aetiology and pathogenesis of Chronic Obstructive Pulmonary Disease (C.O.P.D.) of horses.

1.1. THE DISEASE

C.O.P.D. in the horse refers to a single pulmonary disease (McPherson et al., 1978) in contrast to the more general usage of the term in human medicine to describe a group of different chronic pulmonary diseases with similar functional disturbances. Affected horses commonly show decreased work performance along with chronic coughing, increased respiratory sounds and a chronic nasal discharge (McPherson et al., 1978). Respiratory function studies (Gillespie and Tyler, 1969; Sasse, 1971; McPherson et al., 1978) have shown these animals to have a normocapnic hypoxaemia and increased maximum intrapleural pressure changes, the latter being partly reversed by atropine (Obel and Schmiterlow, 1948). Although alveolar emphysema has been described as a major pathological lesion in C.O.P.D. (Cook and Rossdale, 1963; Gillespie and Tyler, 1969), a recent pathological study of horses affected with C.O.P.D. as defined by McPherson et al., (1978) has shown that diffuse bronchiolitis and alveolar overinflation are the major lesions in the disease, with some limited pulmonary emphysema in only a few of the most severely affected animals (Nicholls, 1978).

The aetiology of the disease is still the subject of study. However, Cook (1965) and Eyre (1972b) have postulated the involvement of hypersensitivity-type reactions, and more recently McPherson et al., (1978, 1979a) have reported an association of the disease with aerogenous exposure to fungal antigens of the type found in moulding hay, particularly those of Micropolyspora faeni and Aspergillus fumigatus. Nicolet and Bannerman (1975) have shown that the former organism produces chymotrypsin-like antigens during the moulding process, and precipitins against these protease antigens are commonly found in human patients with extrinsic allergic alveolitis (Nicolet and Bannerman, 1975), although the role of these precipitins as the sole immunological factor in the pathogenesis of the disease is in doubt (Schatz, Patterson and Fink, 1977). Lawson et al., (1979) have reported an increased incidence of precipitins against M. faeni in C.O.P.D. affected horses in comparison to healthy controls, and intradermal testing of mould extracts in affected and normal animals by both McPherson et al., (1979a) and Halliwell et al., (1979) have indicated the possible involvement of Type III or Arthus hypersensitivity in the disease. In addition, the successful prophylactic use in C.O.P.D. of the drug disodium chromoglycate, which is believed to act by stabilising mast cell membranes, (Murphy, McPherson and Lawson, 1979) has indicated the possible involvement of a Type I or reagin dependant hypersensitivity

in the disease.

In man, a genetically determined deficiency of the major serum protease inhibitor, alpha-1 antitrypsin, appears to predispose affected individuals to a form of C.O.P.D. associated with panlobular or centrilobular emphysema and marked parenchymal destruction and fibrosis (Eriksson, 1965), and both Gillespie and Tyler (1969) and Breeze (1979) have suggested that a similar predisposition may occur in C.O.P.D. affected horses. However, the dysproteinaemia-related disease in man is pathologically and aetiologically unlike C.O.P.D. in the horse. Nevertheless, the chymotrypsin-like properties of the endogenous proteolytic antigens produced by M. faeni in moulding hay and their possible involvement in the pathogenesis of C.O.P.D., along with the observation that the horse, unlike man, lacks a lung specific protease inhibitor (Von Fellenberg, 1978a; Von Fellenberg, et al., 1979) may indicate that serum antiproteases are important in the defence of the lower airway against direct lysis or hypersensitization by inhaled protease antigens.

1.2. THE PROPOSED INVESTIGATION

These observations on the clinical and pathological features of C.O.P.D. in the horse present two possible lines for investigation of serological factors involved in the onset of the disease. Firstly the role of serum antiprotease deficiency as a possible intrinsic determinant

of the disease, and secondly the importance of humoral hypersensitivity mechanisms in the immunopathogenesis of the disease. Thus, following a preliminary examination of zone electrophoresis as a basic serological tool in the study of C.O.P.D., serum protease homeostasis in the normal horse and its possible association with the onset of C.O.P.D. is investigated in detail. The third section of this thesis describes the preliminary identification and characterisation of a previously undescribed reaginic or homocytotropic antibody in horse serum.

SECTION I

SERUM PROTEIN ELECTROPHORESIS IN THE NORMAL HORSE AND IN HORSES AFFECTED WITH C.O.P.D.

CHAPTER 2

REVIEW OF THE LITERATURE

2.1. SERUM PROTEIN ELECTROPHORESIS

Electrophoresis is the polar movement of charged particles of a colloidal mixture within an electric field (Michaelis, 1909). The electrophoretic mobility (Rf) of an amphoteric particle is a function of it's size, shape and net charge, and of the charge characteristics and internal structure of the supporting medium (Ressler, 1973). Under identical conditions, a mixture will separate in a pattern determined by the Rf of the component particles.

Picton and Linder (1892) first reported the electrophoretic separation of inorganic ions in a buffer solution. This 'moving boundary' technique was improved by Tiselius (1937a), who also introduced a system for the detection and planimetric quantitation of the moving boundaries involving the measurement of changes in the refractive index at a fixed point in the buffer solution. Using this modification, Tiselius (1937b) described the fractionation of human serum into four components; the anodally migrating albumin, alpha and beta globulins and the cathodally migrating gamma globulin.

The 'moving boundary' technique was superseded by technically simpler 'zone' electrophoresis in an inert, solid or semi-solid support media resulting in improved separation due primarily to the elimination of convection phenomena and the introduction of a crude molecular sieving effect. Support media in use are; filter paper (Kunkel and Tiselius, 1952), cellulose acetate

(Kohn, 1957), agar gel (Weime, 1965), agarose gel (Hjerten, 1961), starch gel (Smithies, 1955) and polyacrylamide gel (Raymond and Weintraub, 1959).

In any electrophoretic system, a number of variables will affect the resolution of the protein components, including buffer molarity and pH and electrical running conditions. These factors have been discussed in detail by Ressler (1973) and Sargent and George (1975). Following zone electrophoresis the separated components remain as discrete zones or bands within the supporting medium and may be detected using either specific chromatic staining (Sargent and George, 1975) or immunochemical techniques (Ganrot, 1972), and may be quantitated either directly following elution from the support medium, or by densitometric scanning of the stained electrophoretogram (Sargent and George, 1975).

2.2. SERUM PROTEIN ELECTROPHORESIS IN THE HORSE

Using a moving boundary technique, Tiselius and Kabat (1939) demonstrated the separation of normal horse serum into albumin and alpha, beta and gamma globulin components. Investigations of the physicochemical nature of the immune component of hyperimmune horse serum resulted in the identification of a fifth or 'T' component in hyperimmune antitetanal serum (Tiselius and Kabat, 1939; Van der Scheer, Wyckoff and Clark, 1941), later identified as the unique immunoglobulin class IgG(T) (Weir and Porter, 1966).

Planimetric quantitation of the crude moving boundary electrophoretic fractions of horse serum and plasma was reported by Polson (1943), Deutsch and Goodloe (1945) and Kao, Reagan and Breuckner (1954). Quantitation of the individual fractions after zone electrophoresis of horse serum has been widely reported using a range of techniques and support media:- filter paper (Campbell 1957; Boguth, 1954; Irfan, 1967; Hort, 1968; Sitarska, Wasniewski and Pytowski, 1969; Ferri, et al., 1969; Littlejohn, 1978), cellulose acetate (Mattheeus, et al., 1966; Bierer, 1969; Ek, 1970; Osbaldiston, 1972; Massip and Fumiere, 1974; Pierce, 1975; Kirk, Hutcheson and Neate, 1975; Breeze et al., 1977) and agarose (Morgan, 1972; Liberg, Magnusson and Schougaard, 1977; Kristensen and Firth, 1977). However, variation in the quality of resolution of the protein zones and bands achieved with each electrophoretic system, in particular with the use of different support media, has led to confusing and conflicting published data on the number, identity and nomenclature of the electrophoretic zones. This precludes direct comparison of the results of individual authors.

2.2.1. ELECTROPHORESIS OF HORSE SERUM ON AGAROSE GEL.

Agarose is an almost neutral polysaccharide derivative of agar (Araki, 1956) and its extremely small charge characteristics result in both minimal electroendosmosis

(the net polar flow of gel anions and cations during electrophoresis) and minimal interaction with migrating proteins (Weime, 1964). Both these phenomena diminish the resolution of separated protein. (Weime, 1964; Cann and Goad, 1968).

After agarose electrophoresis (pH 8.6) of horse serum, seven zones, containing a total of nine peaks may be recognised using routine protein staining (Fig. 2.1). Anodally, in order of decreasing Rf, appear a broad albumin zone (A) which may show a distinct shoulder on its cathodal edge (Fig. 2.1b, 2) described as the alpha-1 zone by Kristensen and Firth (1977) but included in the albumin zone by Morgan (1972). The discrete peak appearing in zone B in most horses (Fig. 2.1b, 3) is variably described as the alpha-1 peak (Morgan, 1972; Liberg et al., 1977) or alpha-2a peak (Kristensen and Firth, 1977). The adjacent more cathodal zone (C) commonly shows a distinct peak on its anodal edge (Fig. 2.1b, 4) which has been designated the alpha-2 peak (Morgan, 1972) or the alpha-2b peak (Kristensen and Firth, 1977). Liberg et al., however, did not differentiate two peaks in this zone. The major peak in zone C (Fig. 2.1b, 5) has been identified as the alpha-2 peak (Morgan, 1972) or the alpha-2c peak (Kristensen and Firth, 1977). Both Morgan (1972) and Kristensen and Firth (1977) agreed on the extent and nomenclature of zone D, the beta-1 zone, although Liberg et al., (1977) extended the beta-1 zone towards the cathode to include

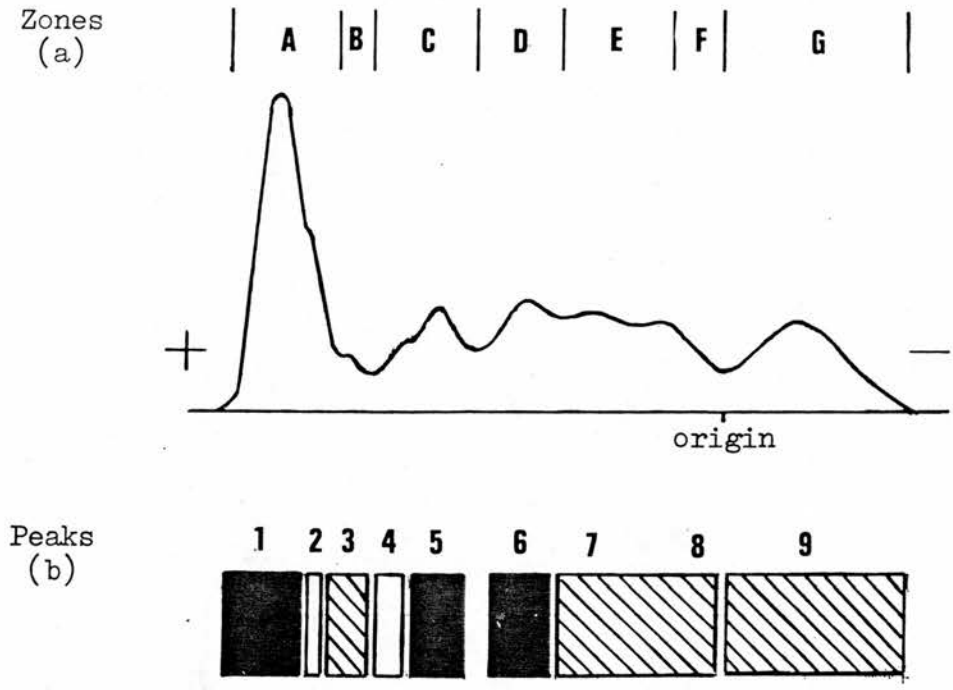


Fig. 2.1. Schematic representation of the agarose electrophoretogram of horse serum after routine protein staining.

(a) Densitometric tracing showing the major electrophoretic zones A - G.

(b) Stained gel showing the individual protein peaks 1 - 9.

electrophoretically slower transferrin variants.

Morgan (1972) and Kristensen and Firth (1977) described zone E as the beta-2 zone, the cathodal limit of which was also recognised by Liberg, et al., (1977). The discrete zone F, resulting from artificial division of the gamma zone by the insertion slit in the gel, was described as the beta-3 peak by Morgan (1972) but was included along with the cathodal protein component (G) in the gamma globulin zone by Liberg, et al., (1977) and Kristensen and Firth, (1977).

Only Morgan (1972), in his study of foals up to 140 days old, recognised a prealbumin fraction in the extreme anodal region of horse serum.

2.2.2. ELECTROPHORETIC DISTRIBUTION OF HORSE SERUM PROTEINS

In the horse, only a limited amount of information has been published on the distribution of individual serum proteins after agarose electrophoresis. Electrophoresis and autoradiography of ^{59}Fe labelled serum has identified transferrin as the major constituent of the beta-1 zone (Morgan, 1972), and both Morgan (1972) and Liberg, et al., (1977) have shown that individual variation in the beta-2 zone can result from the electrophoretic heterogeneity of genetically determined transferrin variants (Braend and Stormont, 1964). Electrophoresis of the void volume of horse serum after Sephadex G200 chromatography (Vaerman, Querinjean and Heremans, 1971) has identified the major constituents of the alpha-2

and gamma-1 zones as alpha-2 macroglobulin and IgM respectively. Similarly, electrophoresis of the low molar eluate of horse serum after anion exchange chromatography has identified the major constituent of the gamma-2 zone as IgG (Vaerman, et al., 1971), and immunoelectrophoresis using monospecific anti-immunoglobulin sera has shown that IgG(T) migrates predominantly in the beta-2/gamma-1 zone (Vaerman, et al., 1971).

2.2.3. PHYSIOLOGICAL FACTORS AFFECTING THE HORSE SERUM PROTEIN ELECTROPHORETOGRAM

(i) Age

A number of authors have investigated the effect of nursing on the serum protein electrophoretogram of the newborn foal (Polson, 1943; Sitarska et al., 1969; Morgan 1972) and, in the most comprehensive study, Morgan (1972) reported a rise in total protein during the first 72 hours post partum associated with a rise in the albumin and gamma globulin fractions. Between five days and five months post partum both Sitarska, et al. (1969) and Morgan (1972) reported a transient fall in the total protein, albumin and gamma globulin concentrations followed by a gradual increase towards adult levels. Morgan (1972) also described a significant alpha-2a peak (Fig. 2.1b, peak 4) in foals up to 28 days old.

Although Massip and Fumiere (1974) reported the

results of a quantitative study of the serum electrophoretic patterns of 98 adult horses aged between four and ten years, grouping their data according to age, they did not carry out statistical analysis of the variance between each group. Hence, the significance of differences between the groups was not established. However, a significant correlation ($0.01 < P < 0.05$) of increasing gamma globulin concentration with age up to five years has been established by Kristensen and Firth (1977).

(ii) Sex

A number of authors have investigated the influence of sex on the serum electrophoretogram (Ferri et al., 1969; Massip and Fumiere, 1974; Kirk, et al., 1975; Pierce, 1975). However, no significant intersex differences in the levels of total serum protein or individual protein fractions have been reported.

(iii) Breed

Although Hort (1968) was unable to demonstrate significant differences between the serum electrophoretic fraction concentrations of six horses and four ponies, Kirk et al., (1975) reported raised mean total protein, alpha-2 globulin and beta-2 globulin levels in 7 healthy ponies in comparison to 14 healthy horses. Breeze, et al., (1977) subsequently demonstrated significant differences ($0.05 > P > 0.02$) in the mean albumin and total beta globulin levels between two groups of 16 ponies and 28 horses showing no signs of respiratory disease,

the former being relatively hypoalbuminaemic and hyper-beta globulinaemic. However, no significant differences were found between 9 horses and 10 ponies affected with C.O.P.D. as defined by McPherson et al., (1978).

Kristensen and Firth (1977) described a discrete alpha-1 band of Rf midway between the alpha 1a and alpha-1 peaks, which was present in all Arab horses they examined.

(iv) Bodily Condition

Pierce (1975) arbitrarily divided 104 clinically normal animals from one herd into four groups according to general bodily condition; viz, obese, very good, good and thin. Between these groups, no significant differences in the levels of total protein or individual electrophoretic fractions were observed.

2.2.4. CLINICAL APPLICATION OF SERUM PROTEIN ELECTROPHORESIS IN THE HORSE.

Although individual serum proteins will vary independantly of one another in health and disease (Laurell, 1973), quantitative serum protein electrophoresis has been used as a diagnostic aid in equine medicine solely to identify a number of non-specific changes associated with certain diseases. These are:- hypoalbuminaemia associated with hepatic disease (Coffman 1969; Jeffcott, 1971), severe parasitism (Ooms et al., 1976) and some forms of chronic enteritis (Merritt et al., 1977), hypoproteinaemia associated with malabsorption (Meunten et al., 1978), and hyperglobulinaemia associated with chronic suppurative

infection (Coffman, 1969; Jeffcott 1971; Rumbaugh, Smith and Carlson, 1978). A specific increase in the alpha-2 and beta fractions has been demonstrated in experimental prepatent strongyliasis, and has been suggested to be in part due to an IgG(T) mediated immune response to somatic or metabolic parasitic antigens (Round, 1969, 1971; Amborski, Bello and Torbert, 1974). Allegedly increased alpha-2 and beta-2 fractions have also been reported in two ponies with laminitis (Kirk et al., 1975).

Quantitative serum protein electrophoresis has been widely used in human clinical medicine for a number of years (Ritchie, 1969), and Werner, Brooks and Cohnen (1972) have shown that electrophoresis, interpreted with the help of multivariate analysis, can provide diagnostic information equivalent to that of a limited set of specific serum protein assays. Nevertheless, Laurell (1973) argued that qualitative interpretation of the electrophoretogram accompanied by specific serum protein assays will provide the maximum amount of clinically useful data.

2.3. CONCLUSIONS FROM THE LITERATURE SURVEY

It is apparent from the diversity of published data that no author has attempted to objectively standardise the divisions and nomenclature of the horse serum protein electrophoretogram. Such standardization is necessary before significant comparisons of the electrophoretic profiles of healthy and diseased horse populations or

individuals may be drawn. In the absence of readily available and specific horse serum protein assays, maximum information will be obtained from quantitative serum protein electrophoresis where the divisions or zones are based upon the distribution of individual proteins, such that variation in each zone may be related to variation in a single protein or group of proteins.

Furthermore, the wide variation in electrophoretic profiles of apparently clinically normal horses and ponies indicates that the relative concentration of individual protein components is subject to considerable physiological, genetic or environmental variation. Such variations must be recognised before attempting to compare data derived from normal and diseased populations.

CHAPTER 3

THE SERUM ELECTROPHORETIC PROFILES OF CLINICALLY NORMAL HORSES AND THE EFFECT OF PHYSIOLOGICAL VARIATION.

3.1. INTRODUCTION

The establishment of quantitatively useful divisions of the horse serum protein electrophoretogram is dependant upon the identification of the distribution of those protein components which by individual variation in concentration may significantly alter the electrophoretic profile (2.3). Laurell (1972a) has stated that quantitative variation in plasma proteins whose minimum concentration lies between 0.1 and 0.5 g/l may significantly alter the serum electrophoretic profile. Thus,as the lower limits of the normal serum concentration of transferrin, alpha-2 macroglobulin and the immunoglobulins G, M and G(T) are in excess of this threshold level (Makimura et al., 1975; Lavergne and Raynaud, 1970; McGuire, Crawford and Henson, 1972), then variation in the circulating levels of these proteins will be reflected in the electrophoretic profile. Other proteins which by virtue of their normally high serum concentrations may significantly affect the serum electrophoretic profile are albumin, haptoglobin (Allen and Archer, 1971), the lipoproteins (Robie, Smith and O'Connor, 1975) and in the neonatal and perinatal foal, alphafetoprotein (Lock, Morgan and Mock, 1976).

In this chapter, the Rf of these proteins was used to define the electrophoretic zones of horse serum as a baseline for the subsequent investigation of quantitative variation in the electrophoretic profile associated with

selected physiological parameters:- sex, breed and age over 1 year.

Because of its high resolution properties (2.2.1.), an agarose gel electrophoresis system was used throughout this study.

3.2. MATERIALS AND METHODS

3.2.1. ELECTROPHORESIS

A commercially available agarose electrophoresis system[1], employing an agarose (1% $^{w}/v$) - sucrose (5% $^{w}/v$) gel in 0.05M barbital-EDTA buffer (0.035% ($^{w}/v$) EDTA, pH 8.6), and a continuous gel-electrode buffer system was used throughout. Following the application of 0.8 µl serum to precut troughs on the gel plate, electrophoresis was carried out at 100V for 21 minutes. The system permitted the simultaneous electrophoresis of eight samples.

Routine protein staining was carried out using 0.2% ($^{w}/v$) Amido-Black B in 5% ($^{v}/v$) glacial acetic acid, followed by destaining in 5% ($^{v}/v$) glacial acetic acid according to the procedure detailed in the Corning-EEL

1 Corning-EEL, Palo Alto, California, 94306.

electrophoresis manual.

The relative protein composition of the individual electrophoretic zones was determined as percentage optical absorbance at 520 nm using a Phoroscope Densitometer[1], and the absolute concentration (g/L) calculated from the total serum protein determined using the standard Biuret method of Henry, Sobel and Berkman (1957).

In a study of the reproducibility of this system, Keay (1978, pers. comm.) was unable to demonstrate significant interplate plate variation in electrophoretically determined serum albumin levels ($0.01 < P < 0.05$). However, experimental variability associated with the densitometric scanning procedure was determined as the coefficient of variation (CV) of eight consecutive scannings of the same electrophoretic run, where $CV = \frac{100 \times S.D.}{\bar{x}}$. (Sokal and Rohlf, 1973).

3.2.2. SERA

Pony serum was obtained from two clinically normal populations maintained independently on islands off the Scottish mainland. A total of 47 individual sera were collected from 3 stallions, 4 geldings and 40 mares, all aged 1 year or older.

Horse serum was obtained from three types of clinically normal animals; Thoroughbreds, Heavy Hunters and Trotting Horses. A total of 30 individual sera were collected from 2 stallions, 18 geldings

1 Millipore Corporation, Bedford, Mass., U.S.A.

and 10 mares, all aged 1 year or older.

3.2.3. ELECTROPHORETIC DISTRIBUTION OF SELECTED SERUM PROTEINS.

To supplement previously published data on the electrophoretic distribution of transferrin, alpha-2 macroglobulin and the immunoglobulin classes M, G and G(T) (2.2.2.), the distribution of haptoglobin, lipoproteins and alphafetoprotein after agarose electrophoresis was determined. In addition, statistical comparison of electrophoretically and biochemically measured albumin concentration of horse serum assessed the accuracy of the electrophoretic albumin zone limits used in this study.

(i) Haptoglobin

The electrophoretic mobility of both haptoglobin-haemoglobin (Hp - Hb) complexes and free haemoglobin (Hb) was determined using an O-dianisidine/hydrogen peroxide staining method as described in the Corning-EEL electrophoresis manual. A solution of horse haemoglobin was prepared by the hypotonic lysis of packed, thrice washed, heparinized blood and was added to serum in a ratio of 1:7 30 minutes before electrophoresis. Paired samples with and without haemoglobin were simultaneously electrophoresed and the latter were stained routinely with Amido-Black B.

(ii) Lipoproteins

Paired samples of the same sera were simultaneously electrophoresed and stained with Amido-Black B and Fast Red 7B respectively. The latter procedure was carried out as described in the Corning-EEL electrophoresis manual.

(iii) Alphafetoprotein

Serum from two presuck foals and their respective dams were simultaneously electrophoresed and the alphafetoprotein component was identified after Amido-Black B staining by reference to the data of Lock, et al., (1976).

(iv) Albumin

To determine the accuracy of the electrophoretic albumin zone used throughout this study, the albumin levels of 20 sera from the horse population were determined electrophoretically and biochemically using the bromocresol green (BCG) dye binding method of Doumas, Watson and Biggs (1971). The BCG method employed a 10 minute reaction time as used routinely in the clinical laboratory in the Department of Veterinary Medicine of the University of Edinburgh. The electrophoretic albumin zone in the present study excluded the cathodal shoulder region of the albumin peak.

The t-test of paired comparisons (Sokal and Rohlf, 1973) was used to test whether the means of sample differences between paired determinations differed significantly from zero.

3.2.4. THE EFFECT OF PHYSIOLOGICAL VARIATION ON THE ELECTROPHORETIC PROFILE OF HORSE SERUM.

Following definition of the electrophoretic zones of horse serum, the effect of physiological variation on these zones was examined. Statistical comparisons between the mean protein concentration of the electrophoretic zones of the horse and pony populations, and subgroups of the horse population segregated according to sex and age were carried out using Student's 't' test and F-distribution analysis of variance (Smart, 1970).

3.3. RESULTS

3.3.1. AGAROSE ELECTROPHORESIS OF HORSE SERUM

The typical distribution of horse serum proteins after agarose electrophoresis (pH 8.6) is shown in Figure 3.1.

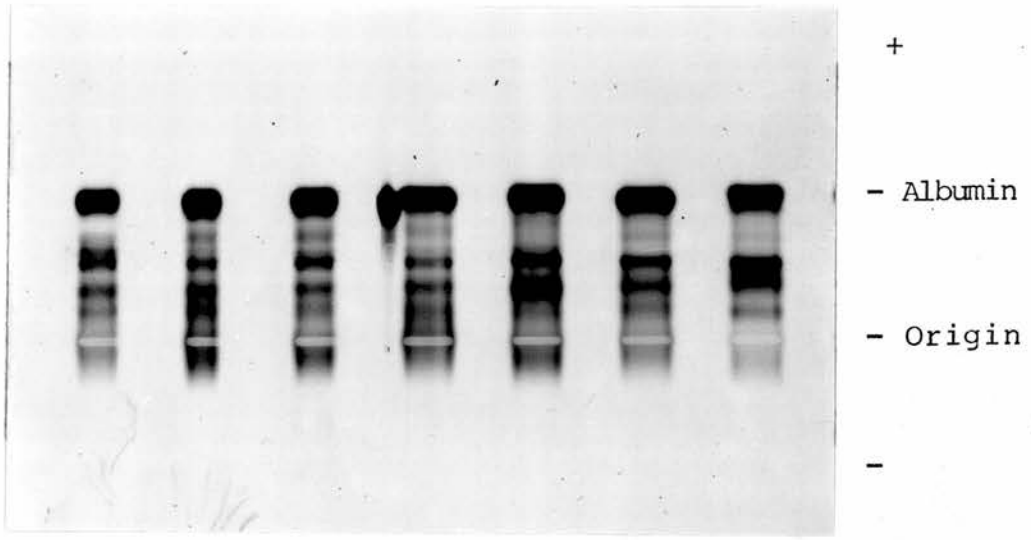


Fig. 3.1. Agarose electrophoretogram of horse serum, stained 0.2% (^{w}/v) Amido-Black B.

3.3.2. THE ELECTROPHORETIC DISTRIBUTION OF INDIVIDUAL SERUM PROTEINS

(i) Haptoglobin

The densitometric profile of the serum-haemoglobin incubate after O-dianisidine/hydrogen peroxide staining of the Hp-Hb complex and free Hb is shown in Figure 3.2b. The relatively cathodal position of free Hb indicates

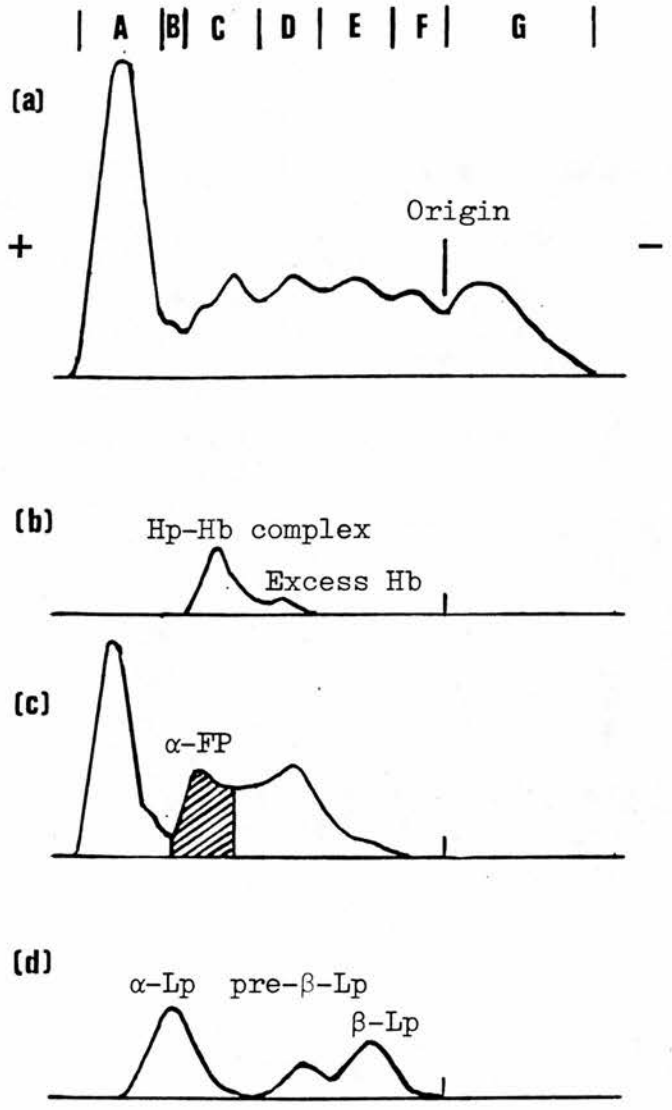


Fig. 3.2. Diagramatic representation of (a) the densitometric tracing of horse serum after agarose electrophoresis, and the distribution of (b) Haptoglobin-haemoglobin complexes (c) alphafetoprotein (α-FP) and (d) the lipoproteins.

that as in the case of human Hp 1-1 type uncomplexed Hp will migrate in the anodal shoulder region of zone C. Phenotypic variation in horse haptoglobin after starch gel electrophoresis has not been recorded (Braend and Effremov, 1965), and variation in this C zone mobility is not expected.

(ii) Lipoproteins

The densitometric profile of the horse serum electrophoretogram after Fast Red 7B staining is shown in Figure 3.2d. As in man, three lipoprotein peaks are apparent which according to their electrophoretic mobility are designated alpha, pre-beta and beta. In horse serum the alpha, pre-beta and beta lipoproteins migrate in zones B, D and E/F respectively.

(iii) Alphafetoprotein

The typical electrophoretic profile of presuck foal serum is shown in Figure 3.2c. The hatched area is the neonatal globulin component corresponding to alphafetoprotein (Lock, et al., 1976).

(iv) Albumin

The mean $\pm$ 1 S.D. serum albumin concentrations in a population of 20 horses, measured electrophoretically and biochemically are shown in Table 3.1. The individual serum concentrations are presented in Appendix I. Statistical analysis of the data using the t-test of paired comparisons is presented in Table 3.1.

The mean of the sample differences between the paired values does not differ significantly from zero

TABLE 3.1.

(a) THE MEAN $\pm$ 1 S.D. ALBUMIN CONCENTRATION (g/L) OF 20 HORSE SERA DETERMINED ELECTROPHORETICALLY AND BIOCHEMICALLY.

MEAN $\pm$ 1 S.D. SERUM ALBUMIN CONCENTRATION (g/L)	
Measured Electrophoretically	Measured Biochemically
31.29 $\pm$ 2.73	32.34 $\pm$ 4.03

(b) T-TEST OF PAIRED COMPARISONS APPLIED TO THE MEAN OF SAMPLE DIFFERENCES BETWEEN THE PAIRS OF DETERMINATIONS.

T-TEST OF PAIRED COMPARISONS
Mean difference between paired determinations = 0.95
t_s = 1.2376
d.f. = 19
$P > 0.05$

($P > 0.05$), indicating that the mean population serum albumin concentrations derived by electrophoresis is comparable to that derived using a BCG dye binding technique in routine clinical laboratory use.

3.3.3. THE ELECTROPHORETIC ZONES OF HORSE SERUM

Using the above data in conjunction with previously published data (2.2.2), a composite diagram of the electrophoretic distribution of the major serum proteins was compiled (Fig. 3.3). Based upon this distribution, and using the original nomenclature of Tiselius and Kabat (1939), seven electrophoretic zones were defined (Fig. 3.3) such that quantitative variation in each zone could be related to variation in the individual protein components.

The albumin zone excludes the cathodal shoulder region on the albumin peak, which is included within the alpha-1 zone. The alpha-1 zone contains predominantly alpha lipoprotein. The variably biphasic alpha-2 zone in the adult contains predominantly haptoglobin and, more cathodally alpha-2 macroglobulin. In the neonate, alphafetoprotein is the major component of the alpha-2 zone. The beta-1 zone contains predominantly transferrin and pre-beta lipoprotein, and the beta-2 zone contains the electrophoretically slower transferrin phenotypes, beta lipoprotein and IgG(T).

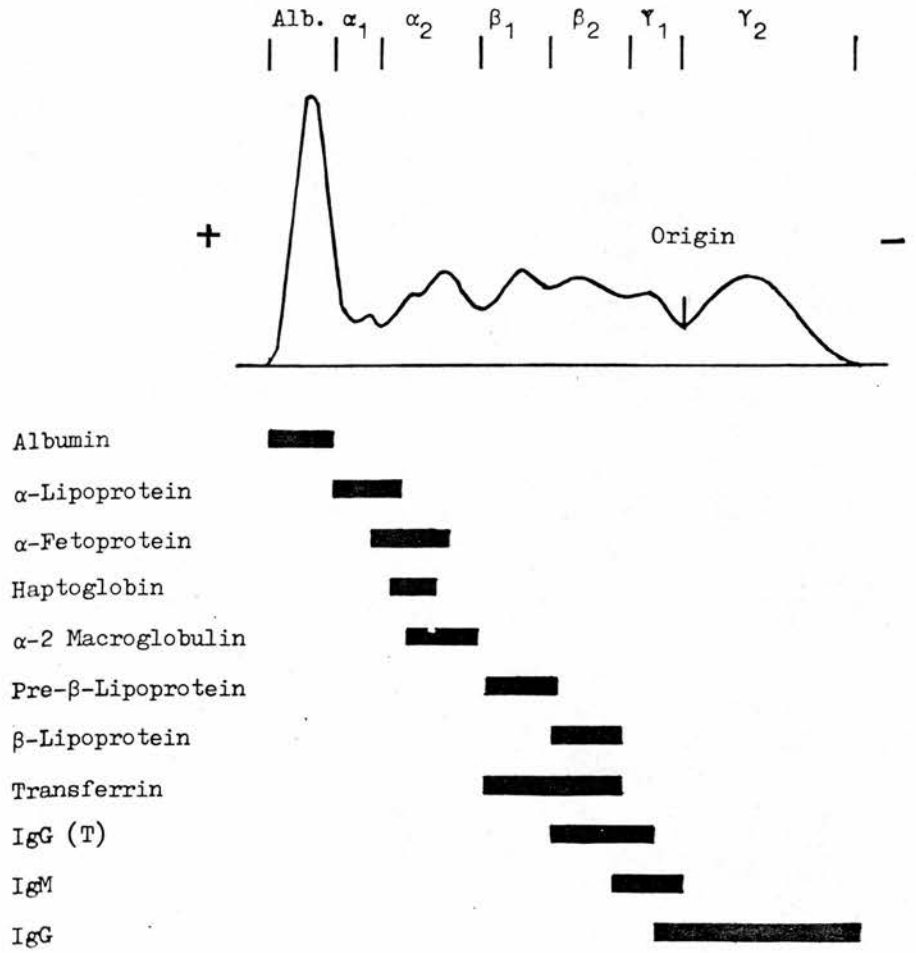


Fig. 3.3. Diagramatic representation of the electrophoretic distribution of some major serum proteins, and the delineation of the electrophoretic zones derived from this distribution.

The gamma zone is artificially divided by the insert slit into the gamma-1 and gamma-2 zones. The gamma-1 zone contains the anodally migrating immunoglobulins M, G (T) and, though not shown in Figure 3.3, immunoglobulin A (Vaerman et al., 1971). The gamma-2 zone contains predominantly the cathodally migrating immunoglobulin G.

The mean $\pm$ 1 S.D. of the electrophoretic mobilities (Rf) of the anodal zones are shown in Table 3.2. The Rf is expressed as $\frac{dz}{da}$, where dz is the distance of migration (mm) of the major peak within each zone and da is the distance of migration (mm) of the albumin peak (Osbaldiston, 1972).

TABLE 3.2.

Rf (MEAN $\pm$ 1 S.D.) OF THE ELECTROPHORETIC ZONES OF HORSE SERUM.

Electrophoretic Zone	Rf $\bar{x}$	S.D.
Albumin	1	
Alpha-1	0.775	$\pm$ 0.018
Alpha-2	0.582	$\pm$ 0.010
Beta-1	0.411	$\pm$ 0.020
Beta-2	0.231	$\pm$ 0.030
Gamma-1	0.061	$\pm$ 0.011

3.3.4. EXPERIMENTAL VARIATION ASSOCIATED WITH DENSITOMETRIC SCANNING OF THE STAINED ELECTROPHORETOGRAM.

The mean $\pm$ 1 S.D. of the percentage composition of the individual zones derived from eight consecutive scans of the same electrophoretic run are shown in Table 3.3.

The coefficient of variation (CV) estimates the reproducibility of the scanning procedure, and the inverse correlation of CV with protein concentration indicates that increased subdivision of the basic electrophoretic zones may introduce significant error into the quantitative scanning procedure.

TABLE 3.3.

REPRODUCIBILITY OF DENSITOMETRIC SCANNING PROCEDURE. THE MEAN $\pm$ 1 S.D. PERCENTAGE COMPOSITION OF EACH ZONE DERIVED FROM EIGHT CONSECUTIVE SCANS OF THE SAME ELECTROPHORETIC RUN, AND THEIR RESPECTIVE COEFFICIENTS OF VARIATION.

Electrophoretic Zone	Percentage Composition $\bar{x}$ S.D.	Coefficient of variation-CV (%)
Albumin	47.5 $\pm$ 1.93	4.1
Alpha-1	9.625 $\pm$ 0.86	8.9
Alpha-2	9.125 $\pm$ 0.78	8.5
Beta-1	16.125 $\pm$ 0.78	5.1
Beta-2	7.625 $\pm$ 0.74	9.7
Gamma-1	2.5 $\pm$ 0.5	20
Gamma-2	7.625 $\pm$ 0.70	9.1

3.3.5. THE EFFECT OF PHYSIOLOGICAL VARIATION ON THE ELECTROPHORETIC PROFILE OF HORSE SERUM.

The total serum protein concentration and the absolute protein concentration (g/l) of the electrophoretic zones of the individual animals within the horse and pony populations are presented in Appendix 2. The age and sex of the individuals within the horse population are also presented. Their respective means $\pm$ 1 S.D. along with statistical analyses of the differences between the two populations is shown in Table 3.4a. Similarly, statistical analyses of differences between the mean zone protein concentrations of the geldings and mares within the horse population is shown in Table 3.4b. The analyses of variance between the mean zone protein concentration of three sub groups of the horse population segregated according to age are shown in Table 3.4c. These three sub-populations are A (animals aged 2 - 6 years), B (animals aged 7 - 9 years) and C (animals aged more than 9 years). This segregation permits the inclusion of statistically significant numbers ($n \geq 8$) within each group.

The data presented in Table 3.4a show that with the exception of the gamma-1 fraction marked differences occur between the serum electrophoretic zone concentrations of normal horses and ponies. No significant differences were observed between the serum electrophoretic zone concentrations of the geldings and

TABLE 3.4

THE EFFECT OF PHYSIOLOGICAL VARIATION ON THE HORSE SERUM PROTEIN ELECTROPHORETOGRAM. STATISTICAL COMPARISON OF THE MEAN ELECTROPHORETIC ZONE COMPOSITION (g/l) OF ANIMALS SEGREGATED ACCORDING TO (a) BREED, (b) SEX AND (c) AGE.

(a) Breed

Electrophoretic Zones	POPULATIONS		t (d.f.=75)	Significance 'P'
	Horse $\bar{x}$ n=30 S.D.	Pony $\bar{x}$ n=47 S.D.		
Total protein	65.85 ± 6.64	70.25 ± 7.90	2.6307	$0.005 < P < 0.01$
Albumin	29.89 ± 3.39	25.05 ± 3.67	5.9146	$P < 0.001$
Alpha-1	6.40 ± 1.21	5.24 ± 1.00	4.3819	$P < 0.001$
Alpha-2	6.72 ± 1.84	9.88 ± 3.32	5.3615	$P < 0.001$
Beta-1	9.01 ± 3.30	12.65 ± 2.77	5.0177	$P < 0.001$
Beta-2	5.39 ± 1.60	7.85 ± 2.00	5.9587	$P < 0.001$
Gamma-1	2.22 ± 0.71	2.20 ± 0.69	0.1219	n.s.*
Gamma-2	6.33 ± 1.54	7.65 ± 2.26	3.0466	$P < 0.001$

* n.s. = not significant, $P > 0.05$.

d.f. = degrees of freedom.

TABLE 3.4 (Continued)

THE EFFECT OF PHYSIOLOGICAL VARIATION ON THE HORSE SERUM PROTEIN ELECTROPHORETOGRAM.

STATISTICAL COMPARISON OF THE MEAN ELECTROPHORETIC ZONE COMPOSITION (g/L) OF ANIMALS SEGREGATED ACCORDING TO (a) BREED, (b) SEX AND (c) AGE.

(b) Sex

Electrophoretic Zones	HORSE POPULATION				t (d.f.=26)	Significance 'P'
	Geldings $\bar{x}$	n=18 S.D.	Mares $\bar{x}$	n=10 S.D.		
Total protein	64.43 ±	5.49	67.33 ±	8.12	1.0086	n.s.*
Albumin	30.31 ±	2.96	28.16 ±	3.39	1.6809	n.s.
Alpha-1	6.36 ±	1.22	6.46 ±	1.19	0.2111	n.s.
Alpha-2	6.37 ±	1.74	7.13 ±	1.46	1.2307	n.s.
Beta-1	8.26 ±	2.62	10.46 ±	3.86	1.6082	n.s.
Beta-2	5.03 ±	1.72	6.12 ±	1.31	1.8805	n.s.
Gamma-1	2.21 ±	0.76	2.27 ±	0.73	0.2053	n.s.
Gamma-2	6.09 ±	1.47	6.70 ±	1.79	0.9191	n.s.

*n.s. = not significant, $P > 0.05$. d.f. = degrees of freedom.

TABLE 3.4 (Continued)

THE EFFECT OF PHYSIOLOGICAL VARIATION ON THE HORSE SERUM PROTEIN ELECTROPHORETOGRAM. STATISTICAL COMPARISON OF THE MEAN ELECTROPHORETIC ZONE COMPOSITION (g/l) OF ANIMALS SEGREGATED ACCORDING TO (a) BREED, (b) SEX AND (c) AGE.

(c) Age

Electrophoretic Zones	HORSE POPULATION			Analysis Of Variance 'F' **	Significance 'P'
	Group A, n = 9 (2 ± 6 year old) $\bar{x}$ S.D.	Group B, n = 12 (7±9 year old) $\bar{x}$ S.D.	Group C, n = 9 (≥10 year old) $\bar{x}$ S.D.		
Total protein	67.94 ± 5.38	65.46 ± 8.01	64.27 ± 4.51	2.4711	n.s. *
Albumin	31.03 ± 2.41	27.75 ± 2.83	31.60 ± 3.22	5.1752	$0.01 < P < 0.05$
Alpha-1	6.74 ± 0.98	6.22 ± 1.22	6.30 ± 1.25	0.5209	n.s.
Alpha-2	7.66 ± 1.80	6.52 ± 1.79	6.05 ± 1.41	1.9430	n.s.
Beta-1	8.84 ± 3.12	10.19 ± 3.49	7.60 ± 2.32	1.6718	n.s.
Beta-2	5.78 ± 1.83	5.67 ± 1.45	4.62 ± 1.10	1.5507	n.s.
Gamma-1	2.09 ± 0.65	2.32 ± 0.65	2.23 ± 0.78	0.2527	n.s.
Gamma-2	5.87 ± 1.17	6.80 ± 1.71	6.17 ± 1.35	1.0447	n.s.

*n.s. = not significant, $P > 0.05$.
**d.f. = degrees of freedom; $V_1 = 2$, $V_2 = 27$.

mares within the horse population. Within the horse population however, the serum albumin concentrations was significantly lower in group B (7 - 9 years old), although no other significant age related changes in serum electrophoretic zone concentration were observed.

3.4. DISCUSSION

Objective definition of the electrophoretic zones of horse serum according to the distribution of some major serum proteins has established a standard for future interpretation of the agarose derived electrophoretic pattern. Furthermore, changes in zone concentrations may be related to changes in the constituent proteins thus increasing the diagnostic potential of the technique.

The results of this study have shown that the

electrophoretically estimated serum albumin levels compare favourably with those determined using a standard dye binding procedure. In a study of bovine serum protein electrophoresis, Liberg (1977) suggested that electrophoresis underestimated the true serum albumin concentration, and this presumption was extended to a subsequent study of horse serum protein electrophoresis by Liberg et al., (1977) who stated that owing to the staining intensity of the albumin peak the electrophoretically determined serum albumin levels would be insufficiently accurate. These authors then used the standard dye binding technique of Doumas et al., (1971) to estimate serum albumin levels and referred to the serum electrophoretic profile solely to determine the relative concentration of individual globulin components. However, the biochemically estimated mean serum albumin level reported by Liberg et al., (1977) in their series of 18 trotting horses (32.8 g/l $\pm$ 2.8) compares favourably with the biochemically estimated mean albumin level of the 20 horse sera included in the present series, (32.34 g/l $\pm$ 4.03). The electrophoretically estimated mean percentage albumin level within the horse population in this study, 43.4%, is higher than the 36.2% value recorded by Kristensen and Firth (1977) using the same electrophoretic apparatus and a similar delineation of the stained albumin zone. This discrepancy probably results from the use of a 35 minute running time by these

authors in contrast to the 21 minutes used in the present study, with a consequent dilution effect on the electrophoretically faster protein zones. This effect is illustrated by statistical comparison of the Rfs of corresponding zones in the present study and those of Kristensen and Firth (1977) (Table 3.5). Significant differences in Rf associated with differences in running time, and the consequent quantitative effect on the more anodal zones serve to illustrate that even with a standard basis of interpretation, comparison of quantitative electrophoretic data derived from sources using different techniques is inaccurate.

TABLE 3.5

COMPARISON OF THE MEAN ELECTROPHORETIC MOBILITIES (Rf) OF THE ALPHA AND BETA ZONES IN THE PRESENT STUDY WITH THE CORRESPONDING VALUES OF KRISTENSEN AND FIRTH (1977).

Zones	Rf (present series) n = 12	Rf (Kristiensen and Firth, 1977) n = 50	t d.f.=60	Significance 'P'
alpha-1	0.775 $\pm$ 0.018	0.729 $\pm$ 0.019	7.864	$P<0.001$
alpha-2	0.582 $\pm$ 0.010	0.551 $\pm$ 0.010	9.644	$P<0.001$
beta-1	0.411 $\pm$ 0.020	0.380 $\pm$ 0.018	4.913	$P<0.001$
beta-2	0.231 $\pm$ 0.030	0.204 $\pm$ 0.022	2.934	$0.001<P<0.005$

d.f. = degrees of freedom.

Clinically, low serum albumin levels are encountered in malnutrition (Ricketts and Rossdale, 1975) and debilitating disease (2.2.4). The relative hypoalbuminaemia observed in the clinically normal pony population in the present study (Table 3.4a) is unlikely to be of genetic origin since albumin phenotypes are common to all breeds of horse so far examined (Blokhuis and Buis, 1979), and probably reflects a low plane of nutrition and/or the presence of subclinical disease.

Alpha-lipoprotein is a major component of the alpha-1 zone of human, bovine and equine serum. However in the two former species the broad band of the lipoprotein results in diffuse background staining of the zone (Laurell, 1973; Carlstrom and Liberg, 1975), whereas in the horse the distinct alpha-lipoprotein peak probably forms the variable but discrete alpha-1 peak. The breed-related appearance of twin peaks in the alpha-1 zone reported by Kristensen and Firth (1977) may reflect the occurrence of two alpha-lipoprotein components recorded in Morgan horses, but not Thoroughbreds (Robie, et al., 1975), although Juneja, et al., (1978) have suggested that the bifid alpha-1 peak may result from phenotypic variation at the serum post-albumin locus. The distinct alpha-1 peak in human serum is alpha-1 antitrypsin (Laurell and Eriksson, 1963), however no analagous alpha-1 protease inhibitor has been demonstrated in horse serum (Nakamura and Wakeyama, 1961).

In the adult horse the major components of the alpha-2 zone are haptoglobin and alpha-2 macroglobulin, although in the neonate circulating alphafetoprotein forms a distinct cathodal peak within the alpha-2 zone (Lock et al., 1976). In man, alphafetoprotein has been reported in the serum of adults suffering from hepatocellular carcinoma and gonadal teratoma (Smith, 1970) and also in the serum of pregnant women (Ruoslaliti, Pihko and Seppala, 1974), although it has not been detected in the serum of pregnant mares (Lock et al., 1976). In man and laboratory animals haptoglobin is an acute phase reactant with large increases in serum levels occurring during the acute inflammatory response (Sutton, 1970). A probable acute phase function has been observed associated with horse haptoglobin (Kent, 1980, pers. comm.). Normally raised haptoglobin levels are found in very young foals (Lumsden, Rowe and Mullen, 1980).

Erickson (1975) stated without providing the evidence that equine alpha-2 macroglobulin is an acute phase reactant, although human serum alpha-2 macroglobulin levels remain stable during acute inflammation (Laurell, Jeppsson and Tejler, 1977). Horse alpha-2 macroglobulin has been shown experimentally to bind antigen-antibody precipitates (Lavergne and Raynaud, 1970) and to

neutralise the infectivity of human influenza A_2 virus (Pepper, 1968), and may thus be involved in immunological defence. However, it is not known whether the macroglobulin is capable of a quantitative response to antigen challenge.

The beta-1 zone contains both transferrin and pre-beta lipoprotein, the latter being a relatively minor component (Straub et al., 1975). The electrophoretic mobility of horse transferrin is determined by 10 known autosomal codominant alleles (Scott, 1976), and care is necessary in interpretation of the beta region of the electrophoretic profile since, as suggested by Ek (1970) and subsequently confirmed by Morgan (1972) and Liberg et al., (1977), molecular heterogeneity of transferrin phenotypes can result in considerable variation in the configuration of the beta zone.

Significantly raised beta globulin levels have been reported in the prepatent stages of experimental strongyliasis (Round 1969, 1971; Amborski et al., 1974) and in naturally acquired helminthiasis, with or without concurrent verminous arteritis of the cranial mesenteric artery (Ooms et al., 1976). Round (1969) has presented the serum electrophoretic profiles of experimentally infected animals, which have shown that the rise is primarily associated with the beta-1 globulins. A number of hypotheses have been proposed to explain the rise in serum beta globulin levels in heavily parasitised horses. According to Schotman (1963) the rise is the

result of increased lipolysis associated with insufficient food intake. However, Straub et al., (1975) demonstrated decreased total and component serum lipid levels in four poorly fed ponies in comparison to four well fed ponies, although an earlier and more extensive study by Morris, Zilversmit and Hintz (1972), clearly demonstrated a marked rise in the pre-beta lipoprotein levels associated with prolonged fasting in ponies. It would thus seem feasible that malabsorption resulting from severe parasitism and/or verminous arteritis may result in increased lipid catabolism and increased circulating levels of the pre-beta lipoprotein.

An alternative hypothesis to explain the hyperbetaglobulinaemia has been put forward by Amborski et al., (1974) who demonstrated an increased glycoprotein staining density in the beta zone of serum from horses experimentally infected with Strongylus vulgaris. These authors suggested that a glycoprotein from the tunica intima of the arterial walls is released into the circulation during the perivascular larval migrans stages of prepatent infection. Yet a third explanation of the hyperbetaglobinaemia has been proposed by both Hobart (cited by Round, 1969) and by Amborski et al., (1974) who suggested that the beta component may be identical to IgG(T) and that high levels represent a monoclonal gammopathy associated with an immune response to parasite antigens.

Although no definitive work has appeared in the

literature to explain the origin of the hyperbeta-globulinaemia occurring during strongyliasis in the horse, it is possible that all three above mechanisms may be involved, the relative importance of each mechanism being dependent on the stage of the infection. However, a helminthological survey of one of the pony populations used in the present study (Dixon, Murphy and Matthews, unpublished report) failed to demonstrate significant Strongylus vulgaris infection although the mean beta-1 and beta-2 globulin levels (13.38 $\pm$ 2.68 g/L and 7.90 $\pm$ 2.14 g/L respectively, n = 23) were significantly higher ($t_{\beta 1}$ = 5.3179, $P < 0.001$; $t_{\beta 2}$ = 4.7062, $P < 0.001$) than the corresponding levels in the healthy horse population (Table 3.4a). Furthermore, biochemical studies showed that the majority of the individuals within this pony population were in a state of negative energy balance, indicated by increased circulating levels of β-hydroxybutyrate and acetoacetate, suggesting that lipolysis is the major cause of the increased beta globulin levels in these animals.

The beta-2 zone contains electrophoretically slow transferrin variants, beta-lipoprotein and IgG(T). Moderately raised beta-lipoprotein levels have been reported during fasting (McCullagh, 1978) and may contribute to the hyperbetaglobulinaemia associated with strongyliasis. IgG(T) is identical to the 'T' component demonstrated in antitetanal horse serum by Tiselius and Kabat (1939), and its immunochemical properties have been well documented (McGuire, Crawford and Henson, 1972). Rouse (1971)

reported significantly higher levels of IgG(T) in the serum of pony-type animals maintained outdoors ($\bar{x}$ =705mg/100ml) than in the serum of stabled Thoroughbreds ($\bar{x}$ = 162mg/100ml), and has suggested that this difference may be genetic and/or parasitic in origin. A single case of IgG(T) paraproteinaemia has been reported, associated with malignant lymphoma (Montgomery, et al., 1968).

Although the division of the gamma zones is an artefact resulting from the serum application slit, the division is justified in that each zone contains the cathodally and anodally migrating immunoglobulin classes respectively. Polyclonal gammopathies have been reported in association with chronic infection (Jeffcott, 1971; Rumbaugh et al., 1978), and McGuire Banks and Poppie (1975) have indicated the usefulness of electrophoresis in detecting agammaglobulinaemia due to selective or combined immunodeficiency.

In comparing the effects of age and sex on the electrophoretic profiles of a healthy adult horse population, the sole significant finding was a slightly lowered mean albumin concentration in the middle aged group of animals, although the relevance of this observation to quantitative electrophoretic studies of large populations of mixed ages is doubtful. Marked differences, however, were observed between the electrophoretic zone concentration of clinically normal horses and ponies. In the pony population the raised total

protein and alpha-2, beta and gamma-2 globulin levels, along with lowered albumin and alpha-1 globulin levels, may be related to nutritional status, the presence of subclinical strongyliasis and possibly breed related differences in IgG(T) levels. These results concur with the earlier observations of Kirk et al., (1975) and Breeze, et al., (1977). The former authors reported a relative hyperproteinaemia in their pony population, associated with raised alpha-2 and beta-2 globulin levels, while the latter also reported a significantly lower mean albumin level in a respiratory disease free pony population in contrast to a similarly selected horse population.

The conclusions drawn from these observations on the effect of physiological variation on the equine electrophoretic profile are that mixed populations of horses and ponies must be segregated into individual types before significant comparisons may be made, and these comparisons may then be made only against similarly restricted populations. However, the complex influence of nutrition and the presence of subclinical disease, particularly helminthiasis, on the electrophoretic profile will cause difficulty in interpretation of any differences observed between such restricted populations.

CHAPTER 4

A COMPARATIVE STUDY OF THE ELECTROPHORETIC PROFILES OF CLINICALLY NORMAL AND C.O.P.D. AFFECTED POPULATIONS.

4.1. INTRODUCTION

Both Breeze, et al., (1977) and Littlejohn (1978) reported quantitative studies of the changes in the serum electrophoretic profiles in C.O.P.D. affected horses and ponies in an attempt to identify an association of serum antiprotease deficiency with the disease, analogous to the well documented association of deficiency of the major alpha-1 globulin, alpha-1 antitrypsin, and chronic pulmonary dysfunction in man (Eriksson, 1965). Breeze et al., using a cellulose acetate electrophoresis system, were unable to identify significant differences between the mean total protein, albumin, and alpha, beta and gamma globulin concentrations of horses and ponies affected with C.O.P.D. as defined by McPherson et al., (1978) and horses and ponies showing no clinical signs of respiratory disease. Littlejohn (1978) however, using a paper electrophoresis system, reported significantly lowered mean alpha-1 ($P < 0.001$) and alpha-3 ($P < 0.05$) globulin levels in 12 horses affected with C.O.P.D. in comparison with those of a healthy control population. The alpha-1 and alpha-3 zones identified by Littlejohn correspond to the alpha-1 zone and the more cathodal alpha-2 peak identified in the present study.

In this chapter the agarose electrophoretic profiles of the clinically normal horse and pony populations described in Chapter 3 were compared with those of

C.O.P.D. affected horse and pony populations.

4.2. MATERIALS AND METHODS

4.2.1. SERA

Patient sera was obtained from 15 horses and 15 ponies of varying age and sex, all affected with C.O.P.D. as defined by McPherson et al., (1978).

The clinically normal horse and pony sera previously described (3.2.2.) were used as controls.

4.2.2. ELECTROPHORESIS

Quantitative serum protein electrophoresis was carried out using an agarose system (pH 8.6) as previously described (3.1.2(i)).

Statistical analyses of differences observed between groups was carried out using Students' 't' test (Smart, 1970).

4.3. RESULTS

The absolute concentration (g/L) of total serum protein and the electrophoretic zones of the individual animals within the C.O.P.D. affected horse and pony populations are presented in Appendix 3. Their respective means $\pm$ 1 S.D. are presented in Table 4.1

along with statistical analyses of differences between the two populations.

TABLE 4.1.

STATISTICAL COMPARISON BETWEEN THE MEAN TOTAL SERUM PROTEIN AND ELECTROPHORETIC ZONE CONCENTRATION (g/L) OF HORSE AND PONY POPULATIONS AFFECTED WITH C.O.P.D.

Electrophoretic Zone	C.O.P.D. AFFECTED				t (d.f.=28)	Significance P
	Horses (n = 15)		Ponies (n = 15)			
	$\bar{x}$	S.D.	$\bar{x}$	S.D.		
Total protein	68.46 ±	6.41	71.81 ±	7.20	1.8020	ns*
Albumin	28.82 ±	4.21	27.88 ±	5.50	0.5256	ns
Alpha-1	6.73 ±	0.67	6.34 ±	1.26	1.0584	ns
Alpha-2	7.91 ±	2.17	9.22 ±	2.35	1.5826	ns
Beta - 1	8.45 ±	1.72	9.41 ±	2.57	1.2023	ns
Beta - 2	6.42 ±	1.98	8.14 ±	1.84	2.4645	$0.01 < P < 0.05$
Gamma-1	2.26 ±	0.64	2.26 ±	0.63	0	ns
Gamma-2	7.45 ±	2.69	8.49 ±	2.14	1.1718	ns

* ns = not significant, $P > 0.05$.

d.f. = degrees of freedom.

The absolute concentration (g/L) of total serum protein and the electrophoretic zones of the individual animals within the clinically normal horse and pony populations are presented in Appendix 2. Their respective means ± 1 S.D. have been previously presented in Table 3.4a. Statistical analyses of the differences

TABLE 4.2.

STATISTICAL COMPARISON BETWEEN THE MEAN TOTAL SERUM PROTEIN AND ELECTROPHORETIC ZONE CONCENTRATIONS (g/L) OF (a) HEALTHY AND C.O.P.D. AFFECTED HORSE POPULATIONS AND (b) HEALTHY AND C.O.P.D. AFFECTED PONY POPULATIONS.

(a) HORSES

Electrophoretic Zone	Healthy (n = 30) $\bar{x}$ S.D.	C.O.P.D. affected (n=15) $\bar{x}$ S.D.	t (d.f. = 43)	Significance P
Total protein	65.85 ± 6.64	68.46 ± 6.41	1.2722	ns*
Albumin	29.89 ± 3.39	28.82 ± 4.21	0.8554	ns
Alpha-1	6.40 ± 1.21	6.73 ± 0.67	1.1761	ns
Alpha-2	6.72 ± 1.84	7.91 ± 2.17	1.8216	ns
Beta-1	9.01 ± 3.30	8.45 ± 1.72	0.7482	ns
Beta-2	5.39 ± 1.60	6.42 ± 1.98	1.7493	ns
Gamma-1	2.22 ± 0.71	2.26 ± 0.64	0.1905	ns
Gamma-2	6.33 ± 1.54	7.45 ± 2.69	1.4947	ns

* ns = not significant, $P > 0.05$. d.f. = degrees of freedom.

TABLE 4.2 (Continued)

STATISTICAL COMPARISON BETWEEN THE MEAN TOTAL SERUM PROTEIN AND ELECTROPHORETIC ZONE CONCENTRATIONS (g/L) OF (a) HEALTHY AND C.O.P.D. AFFECTED HORSE POPULATIONS AND (b) HEALTHY AND C.O.P.D. AFFECTED PONY POPULATIONS.

(b) PONIES

Electrophoretic Zone	Healthy (n = 47) $\bar{x}$ S.D.	C.O.P.D. affected (n=15) $\bar{x}$ S.D.	t (d.f.=60)	Significance P
Total protein	70.25 ± 7.90	71.81 ± 7.20	0.7132	ns*
Albumin	25.05 ± 3.67	27.88 ± 5.50	1.8648	ns
Alpha-1	5.24 ± 1.00	6.34 ± 1.26	3.0853	$0.001 < P < 0.005$
Alpha-2	9.88 ± 3.32	9.22 ± 2.35	0.8502	ns
Beta-1	12.65 ± 2.77	9.41 ± 2.57	4.1704	$P < 0.001$
Beta-2	7.85 ± 2.00	8.14 ± 1.84	0.5202	ns
Gamma-1	2.20 ± 0.69	2.26 ± 0.63	0.3137	ns
Gamma-2	7.65 ± 2.26	8.49 ± 2.14	1.3055	ns

* ns = Not signficant, $P > 0.05$. d.f. = degrees of freedom.

between the mean total serum protein and electrophoretic zone concentration of the clinically normal and C.O.P.D. affected horse populations and clinically normal and C.O.P.D. affected pony populations are presented in Tables 4.2a and 4.2b respectively.

4.4. DISCUSSION

Although highly significant differences in the electrophoretic profiles of the clinically normal horse and pony populations used in this study have been previously described (Table 3.4a), these differences were not apparent between the C.O.P.D. affected horse and pony populations (Table 4.1). This observation concurs with those of Breeze et al., (1977) who, though unable to demonstrate significant differences in the electrophoretic profiles of horses and ponies affected with C.O.P.D., demonstrated a relative hypoalbuminaemia and hyperbetaglobulinaemia within the ponies of a mixed horse and pony population described as showing no clinical evidence of respiratory disease. The reason for the absence of marked interbreed differences in electrophoretic profiles amongst the C.O.P.D. affected animals in the present study is not fully understood. However, it may be partly due to differences in the management of the control and C.O.P.D. affected pony populations. The clinically normal control populations were semi-feral and were maintained outdoors on marginal vegetation with little or no supplementary feeding,

whereas the C.O.P.D. affected population, like the clinically normal horse population, were well managed and stabled companion animals. As previously discussed (3.4), differences in plane of nutrition may have resulted in the hypoalbuminaemia and hyperbeta-globulinaemia within the clinically normal pony population in contrast to the clinically normal horse population, while between similarly managed C.O.P.D. affected horse and pony populations only a relatively minor difference in beta-2 zone concentration was observed.

Contrary to Littlejohn's (1978) findings, no significant differences between the electrophoretic profiles of the control and C.O.P.D. affected horse populations were observed in the present study. The significantly increased beta-1 globulin concentration in the control pony population in contrast to the C.O.P.D. affected pony population in the present study probably results from the different management systems rather than the pathological state of the animals. However, the raised mean alpha-1 globulin concentration in the C.O.P.D. affected animals in the pony populations in the present study is in contrast to the lowered mean alpha-1 globulin level reported in C.O.P.D. affected horses in the study of Littlejohn (1978). The cause of the raised alpha-1 globulin levels within the C.O.P.D. affected pony population is not known.

Comparison of the electrophoretic profiles of

clinically normal and C.O.P.D. affected horse and pony populations has shown that no significant differences occur which may be definitively attributed to the pathological state of the animal. The earlier studies of Breeze et al., (1977) and Littlejohn (1978) were designed to investigate a possible aetiological role of serum antiprotease deficiency in equine C.O.P.D. Although zone electrophoresis is useful in detecting individual alpha-1-antitrypsin deficient human patients (Lieberman, et al., 1969) the low incidence (2-8%) of these individuals within a C.O.P.D. affected population (Kueppers and Black, 1974) prohibits the use of mean alpha-1 globulin levels to demonstrate an association of low levels of the antiprotease with the disease. Thus, if an analagous antiprotease deficiency were expected in the horse then mean serum alpha-1 globulin levels would provide little information to support its hypothetical occurrence. More importantly however no alpha-1 antiprotease has been demonstrated in horse serum (Nakamura and Wakeyama, 1961; Fossum, 1970; Erickson, 1975) and alpha-1 globulin levels will provide no information on the antiprotease activity in horse serum.

SECTION 2

THE IDENTIFICATION AND CHARACTERISATION OF THE MAJOR ANTIPROTEASE COMPONENTS IN HORSE SERUM AND AN INVESTIGATION OF THEIR POSSIBLE ROLE IN THE ONSET OF C.O.P.D.

CHAPTER 5

REVIEW OF THE LITERATURE

5.1. Protease inhibitors have been demonstrated in a wide range of organisms, and are implicated in a number of fundamental biological processes, including fertilization, immune defence mechanisms, inflammation, blood coagulation and tissue neoplasia (Heimburger, 1975).

5.2. HISTORICAL ASPECTS

Mammalian serum antiproteinase activity, originally described by Camus and Gley (1897), was subsequently reported to be associated exclusively with the albumin fraction (Landsteiner, 1900; Cathcart, 1904), although Korschum (1902) identified anti-rennin components in both the albumin and euglobulin fractions of horse serum. Interest later focused on human serum anti-proteolysis, with investigation of its basic physio-chemical characteristics (Hussey and Northrop, 1923; Grob 1943) and its variations in malignant disease (Clark, Clifton and Newton, 1948). Shulman (1952), and later Jacobsson (1955), isolated two electro-phoretically distinct protease inhibitors from human serum, the latter establishing a ratio of approximately 1:9 between the trypsin inhibiting activities of the alpha-2 and alpha-1 inhibitors.

TABLE 5.1.

MAJOR PROTEASE INHIBITORS OF HUMAN PLASMA.

INHIBITOR	PLASMA CONC. µmol/l	% TOTAL TRYPSIN INHIBITORY ACTIVITY	*PROTEASE SPECIFICITY	FUNCTION
α-1 antitrypsin	36.0	80	1,2,3,4,7	Limitation of fibrinolysis. Control of inflammatory leucocyte proteases.
α-2 macroglobulin	2.7	15	1,2,3,4,5,7	Limitation of coagulation and fibrinolysis. Control of inflammatory proteases
Inter-α-trypsin inhibitor	3.0	3	1,2	Control of inflammatory leucocyte proteases
α-1 antichymotrypsin	7.0		2,	
Antithrombin III	4.5		1,5	Local limitation of coagulation
C'1-esterase inhibitor	2.5		3,4,6	Activation control of blood coagulation, fibrinolysis, kallikrein and complement.

* 1 = trypsin 2 = chymotrypsin 3 = plasmin 4 = plasma kallikrein 5 = thrombin 6 = C'1-esterase 7 = elastase

Compiled after Heimburger (1972; 1975) and Laurell (1974).

5.3. PLASMA PROTEASE INHIBITORS IN MAN

The protease inhibitory spectrum and physiological functions of the major human plasma protease inhibitors (Table 5.1) have been comprehensively reviewed by Heimburger (1972, 1975). Five additional inhibitors have been identified; a thiol protease inhibitor (Sasaki et al., 1974), inhibitors of clotting factors IIa (Miller-Andersson, Andersson and Borg, 1973) and Xla (Amir, Pensky and Ratnoff, 1972), and two plasminogen activator inhibitors (Hedner, 1973).

It can be seen from Table 5.1 that alpha-1 antitrypsin and alpha-2 macroglobulin are the more important protease inhibitors.

5.3.1. ALPHA-1 ANTITRYPSIN (ALPHA-1 ANTIPROTEASE)

(i) Biochemistry

The alpha-1 inhibitor in human serum was identified as a 3.5S acid glycoprotein (Schultze, Heide and Haupt, 1962) forming the major component of the characteristic alpha-1 band after zone electrophoresis (pH 8.6) (Burtin, 1960; Laurell and Eriksson, 1963). In common with the other plasma antiproteases, with the exception of alpha-2 macroglobulin, the alpha-1 antiprotease competitively inhibits serine proteases with the formation of a stable inhibitor-protease complex (Cohen, 1973a, 1975; Aubry and Beith, 1977) at an equimolar combining ratio (Saklatvala, Wood and White, 1976). Studies on the

interaction of the inhibitor with trypsin (Johnson and Travis, 1975; Cohen, Geczy and James, 1978) suggest that, in accordance with the general enzyme-inhibitor interaction model proposed by Ozawa and Laskowski (1966), interaction with the protease results in the hydrolysis of a peptide bond at a lysyl residue on the inhibitor chain, with consequent acetylation of the catalytically active site of the enzyme. Recently however, Busby, Shida and Gan (1977) have shown that methylation of the lysyl residues on the inhibitor molecule has no effect on its trypsin inhibitory activity, and Johnson and Travis (1978) have indicated that a methionine-serine or methionine-threonine sequence may occur at the inhibitor active site.

(ii) Genetically Determined Molecular Heterogeneity of Alpha-1 Antitrypsin.

Kueppers, Briscoe and Bearn (1964) and Eriksson (1965) described probands and their families, whose biochemically measured plasma trypsin inhibitory capacity (TIC) was distributed in a classical Mendelian segregation pattern, as though determined by dominant and recessive alleles at a single locus. However, electrophoretic variants of the alpha-1 band in individuals with normal TICs (Axelsson and Laurell, 1965; Laurell and Gustavesson, 1967) indicated that multiple codominant allelism probably occurs at the locus.

Using acidic starch gel electrophoresis (pH 4.95) Fagerhol and Braend (1965; 1966) described a polymorphic prealbumin protein apparently defined by three autosomal codominant alleles. The development of immune precipitates after incubation with specific antiserum identified the protein as alpha-1 antitrypsin (Kueppers and Bearn, 1966a; Fagerhol and Laurell, 1967), the latter authors designating the locus Pi (protease inhibitor).

At present the Pi locus is defined by at least 30 codominant alleles (Cox, 1978), designated alphabetically according to the electrophoretic mobility of the allele product on acidic starch gels. The electrophoretic and isoelectric microheterogeneity of the allele products is well established, and has been reviewed in detail by Fagerhol and Laurell (1970), Fagerhol (1972a) and Cox (1978). An extensive population study of Pi phenotypes by Fagerhol and Gedde-Dahl (1969) confirmed that the electrophoretic allele products represent the expression of multiple, fully penetrant, autosomal codominant alleles at a single locus.

A number of surveys have been carried out to establish the Pi allele frequencies in different racial and ethnic groups, and have been reviewed by both Rynbrandt, Ihrig and Kleinerman (1975) and Fagerhol (1976), who have shown that in all populations examined, over 85 percent of individuals are homozygous for the Pi M allele.

Homozygotes of the electrophoretically slowest, PiZ, allele have abnormally low serum alpha-1 antitrypsin levels, with a consequent predisposition to the development of chronic obstructive pulmonary disease (Eriksson, 1965). Recently two rare PiM subvariant phenotypes have also been shown to result in abnormally low serum alpha-1 antitrypsin levels. (Martin, Sesbone, and Ropartz, 1975; Cox, 1975a). A rare null allele at the Pi locus, with no detectable serum alpha-1 antitrypsin, has also been reported (Martin, Vandeville and Ropartz, 1973).

In a large, randomly mating population the Pi locus exhibits Hardy-Weinberg equilibrium (Fagerhol and Gedde-Dahl 1969), possibly maintained by an unresolved heterozygous advantage (Fagerhol, 1972a). Kueppers (1972) described a hypothetical reproductive advantage of 'deficient' over 'normal' Pi phenotypes; proposing that the antiprotease deficiency may facilitate the dissolution of the ovum zona pellucida by acrosomal proteases, with a consequently increased chance of fertilization.

(iii) The Molecular Abnormality Of the PiZ Allele Product

The 'normal' or M variant of alpha-1 antitrypsin is a single chain polypeptide, with four oligosaccaride side chains (Crawford, 1973; Chan and Rees, 1975). Both the M variant (Cox, 1975b; Saklatvala et al.,

1976; Yoshida et al., 1976), and the S variant (Yoshida et al., 1977), possess 6 or 7 sialic acid residues. The 'deficient' Z variant has only three oligosaccaride side chains (Chan and Rees, 1975) with possibly four sialic acid residues (Cox, 1975b; Yoshida et al., 1976). The antiprotease activity of the PiM and PiZ variants is identical (Yoshida et al., 1976), indicating that PiZZ individuals have low levels of normally functionining alpha-1 antitrypsin. Lactoperoxidase labelling studies (Laurell, Nosslin and Jeppsson, 1977) have shown this low level to be the result of a slow rate of molecular biosynthesis.

Neuraminidase treatment of alpha-1 antitrypsin, resulting in progressive molecular desialyation and net charge loss (Gottschalk, 1966), causes a stepwise decrease in the electrophoretic mobility of the Pi allele products (Cox, 1973; Bell and Carrell, 1973), although each product retains an individual mobility after complete desialyation (Crawford, 1973; Yoshida and Wessels, 1978). Consequently, sialic acid composition alone does not determine the electrophoretic microheterogeneity of the allele products and peptide mapping studies of the PiM (Yoshida et al., 1976), PiS (Yoshida et al., 1977) and PiZ (Yoshida et al., 1976) allele products have indicated that the primary determinant of electrophoretic heterogeneity appears to be amino acid substitutions on the polypeptide chain.

Hepatocytes of PiZZ individuals contain within their endoplasmic reticulum a characteristic PAS positive, diastase resistant substance, antigenically similar to alpha-1 antitrypsin (Sharp and Freier, 1972) which has been shown by Jeppsson, Larsson and Eriksson (1975) to be microaggregations of asialo, aglycosyl alpha-1 antitrypsin. Terminal sialic acid residues appear essential for the release of a glycoprotein molecule from the hepatocyte (Pricer and Ashwell, 1971), suggesting that the amino acid anomaly in the PiZ product may prevent the sequential glycosialation of the polypeptide chain and its consequent accummulation within hepatocytes.

Kuhlenschmidt et al., (1974) reported a single case of hepatic sialyltransferase deficiency in a PiZZ individual. However, Eriksson and Larsson (1976) subsequently reported only one such case in a large population of PiZ homozygotes and heterozygotes.

(iv) Pathophysiological Variation in Serum Alpha-1 Antitrypsin Levels

Elevation in plasma antiprotease activity during pregnancy is the result of a specific increase in alpha-1 antitrypsin levels (Ganrot and Bjerre, 1967). A rise in serum alpha-1 antitrypsin levels has also been observed during oestrogen based contraception (Laurell, Kullander and Thorell, 1968), and following steroid therapy (Heidelberger, 1976).

Serum antiprotease activity was also reported to be increased during severe bacterial pneumonia (Ascoli and Bezzola, 1903) and in a number of necrotizing disease processes (Schulman, 1952). Alpha-1 antitrypsin was subsequently shown to be an acute phase reactant protein, increasing markedly in acute infections (Werner and Odenthal, 1967), and tissue trauma (Griffiths, Woodford and Irving, 1977). Increased alpha-1 antitrypsin levels also occur during malignant neoplasia associated with concurrent tissue destruction (Laurell, 1972b).

In PiZZ homozygotes, the pathophysiological response to hormonal or inflammatory stimuli appears normal in relation to the steady state alpha-1 antitrypsin level (Laurell, 1972b).

(v) Alpha-1 Antitrypsin Deficiency and Chronic Obstructive Pulmonary Disease

Eriksson (1965) described the possible association between the hereditary dysproteinaemia of alpha-1 antitrypsin and early onset chronic obstructive pulmonary disease (C.O.P.D.) showing that one percent of C.O.P.D. patients suffered this deficiency. Between 70 and 80 percent of PiZZ individuals develop the disease (Fagerhol and Laurell, 1970), usually between the ages of 60 and 70 years (Fagerhol, 1976). However, some PiZZ individuals develop pulmonary disease during childhood (Talamo et al., 1971), and yet others may show no clinical symptoms of pulmonary disease throughout

their lives, (Fagerhol, 1976). This distribution in the morbidity of the disease in deficient individuals was postulated by Fagerhol (1976) to be the result of Mendelian variation at a hypothetical, independant locus.

The literature on the occurrence of C.O.P.D. in association with alpha-1 antiprotease deficiency has been reviewed in detail by Kueppers and Black (1974), Lieberman (1975) and Heidelberger (1976). The association of the intermediate serum alpha-1 antitrypsin levels of the PiMZ and PiSS individuals and the onset of C.O.P.D. remains controversial, although it is well established that in these individuals exposure to pulmonary irritants, in particular cigarette smoke, results in accelerated pulmonary dysfunction (Cooper et al., 1974).

The pathological and pathophysiological findings in PiZZ associated C.O.P.D. have been detailed by Orell and Mazodier (1972) and Eriksson and Beven (1972) respectively and are summarised in Table 5.2.

A number of additional clinical conditions have been associated with alpha-1 antitrypsin dysproteinaemia, these are:

(i) Neonatal Cirrhosis (Sharp and Frier, 1972).

(ii) Adult cryptogenic cirrhosis (Berg and Eriksson, 1972).

(iii) Hepatocellular carcinoma (Rawlings et al., 1974).

TABLE 5.2

PATHOLOGICAL AND PATHOPHYSIOLOGICAL FEATURES OF ALPHA-I ANTITRYPSIN DEFICIENCY ASSOCIATED CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Pathology:- Orell and Mazodier (1972)

(i) Panlobular or centrilobular emphysema of generalised or predominantly basilar distribution.

(ii) Parenchymal inflammation and fibrosis.

(iii) Chronic bronchitis and right ventricular hypertrophy is a common but not constant finding.

Pathophysiology:- Eriksson and Berven (1972)

(i) Loss of lung recoil

(ii) Reversal of apex to base ventilation/perfusion gradient.

(iii) Marked hypoxia in most instances, but with no concurrent hypercapnia.

(vi) Detection of Alpha-1 Antitrypsin Deficiency in a Population.

Deficient subjects may be identified by quantitative determination of alpha-1 antitrypsin concentration in serum or plasma or by electrophoretic identification of the individual phenotypes.

(a) Estimation of serum or plasma alpha-1 anti-trypsin concentration.

Quantitation of trypsin inhibitory capacity (TIC) of serum or plasma using a controlled substrate-protease reaction: The TIC is expressed as unit mass of trypsin inhibited per unit volume of serum or plasma. A number of trypsin substrates have been used in its determination, including casein (Jacobsson, 1955; Troyer and Moskowitz, 1968), haemoglobin (Grob, 1943; Jacobsson, 1955), gelatin (Hussey and Northrop, 1923), and fibrin (Clark, Clifton and Newton, 1948). Synthetic aromatic esters were evaluated as potential trypsin substrates by Erlanger, Kokowsky and Cohen (1961), although Dyce and Haverback (1960) had earlier used benzoyl arginine-p-nitroanilide (DL-BAPNA) for estimating plasma TIC. Ester substrates in use are benzoyl-arginine-ethylesterhydrochloride (BAEE) (Merry and Davies, 1974) benzoyl DL-arginine-β-napthylamide (BAA) (Schön, Rässler and Alter, 1962) and N tosyl-L-arginine-p-nitroanilide (TAPNE) (Bartik, Chavko and Kasafirik, 1974).

Serum elastase inhibitory capacity has been determined using fluorescein conjugated elastin (Rinderknecht et al., 1968), and recently the elastase-specific synthetic ester N-acetyl-L-alanine p-nitroanilide has been used to characterise protease inhibitors in rat serum (Rosenberg, Roegner and Becker, 1976).

The use of TIC is limited as it measures total serum anti-protease activity, not specifically the activity associated with the alpha-1 antitrypsin component.

Immunochemical estimation of serum or plasma alpha-1 antitrypsin: Using specific anti-serum, alpha-1 antitrypsin may be quantitated by radial immunodiffusion (Dietz, Rubenstein and Hodges, 1974) or by 'rocket' electrophoresis (Adamson and Mathieson, 1972). Because alpha-1 antitrypsin accounts for 80 percent of the total serum TIC, good correlation has been reported between immunochemical measurement and TIC determination using a synthetic ester substrate (Talamo, Langley and Hyslop, 1972; Dietz, et al., 1974).

Electrophoretic measurement of alpha-1 globulin: Good correlation has been reported between the densitometrically determined alpha-1 globulin fraction of human serum and both serum TIC measured using an ester substrate (Lieberman, Mittman and Schneider, 1969) and immunochemically determined alpha-1 antitrypsin concentration (Dunikoski and Kiefer, 1976).

(b) Phenotyping of alpha-1 antitrypsin variants.

Phenotyping permits the visualisation of the alpha-1 antitrypsin allele products, and a number of techniques are in routine use.

Acidic starch gel electrophoresis: A system of discontinuous horizontal acidic starch gel electrophoresis

(pH 4.95) has been described in detail by Fagerhol (1972b). The resolution of the electrophoretically slower allele products may be increased by subsequent crossed electrophoresis (Fagerhol and Laurell, 1967) or by immunofixation (Lieberman and Gaidulus, 1976) using specific anti-alpha-1 antitrypsin serum.

Isoelectric focusing: Electrofocusing in the pH range 3.5-6.0 on sucrose-polyacrylamide gels results in a high degree of resolution of the Pi allele products (Arnaud, Chapuis-Cellier and Creyssel, 1974; Allen, Harley and Talamo, 1974; Allen et al., 1977; Kueppers and Christopherson, 1978). Electrofocusing has been combined with both immunofixation (Arnaud et al., 1977) and crossed electrophoresis (Hoffman and Van den Broek, 1977).

Immunofixation after agarose electrophoresis: Pi variants may be identified by immunofixation after prolonged agarose electrophoresis at both acid (Ritchie and Smith, 1976) and alkaline pH (Johnson, 1976).

5.3.2. ALPHA-2 MACROGLOBULIN

(i) Biochemistry

Alpha-2 macroglobulin is a complex glycoprotein (Bourillon and Razafirmahaleo, 1972) apparently made up of four disulphide linked subunit chains (Harpel, 1973) and able to inhibit a wide range of serine, thiol, acid and metal proteases (Barrett and Starkey, 1973).

The plasma alpha-2 antitrypsin in man (Jacobsson, 1955) was identified as alpha-2 macroglobulin by Mehl, O'Connell and de Groot (1964), and Ganrot (1966b, c) subsequently confirmed the earlier observations of Haverback et al., (1962) indicating that the macroglobulin-protease complex, though having no significant proteolytic activity, retained considerable activity towards synthetic, low molecular weight substrates such as esters.

Barrett and Starkey (1973) published a detailed review on the interaction of proteases with alpha-2 macroglobulin which indicated that the catalytically active site on the macroglobulin-protease complex appeared readily accessible to low molecular weight substrates and inhibitors, but provided limited access to high molecular weight substrates and inhibitors. The data extracted from the literature, combined with their own experimental data, enabled Barrett and Starkey (1973) to propose a hypothesis of the molecular mechanism of the macroglobulin-enzyme interaction. This involved a two-step process with initial cleavage of a critical peptide bond followed by a conformational change which irreversibly traps the enzyme independantly of a covalent bonding mechanism. Macromolecular substrates and inhibitors are sterically hindered from reaching the enzyme, whereas small molecular weight substrates and inhibitors are not so restricted. These

authors refer to their own unpublished electromicroscopic evidence of quaternary structural changes in the human macroglobulin on interaction with the protease.

Barrett and Starkey (1973) reported an equimolar combining ratio of macroglobulin with both trypsin and chymotrypsin although Steinbuch, et al., (1975) subsequently presented evidence of covalent binding of a single porcine pancreatic elastase molecule with both of the disulphide linked polypeptide chains, each of which form a half molecule of the macroglobulin. However, the molar combining ratio and the extent of residual esterase activity are subjects of disagreement and may vary with the protease subclass (Ganrot, 1967; Barrett and Starkey, 1973; Ohlsson and Olsson 1974b, 1977; Twumazi, et al., 1977). Topping and Seilman (1979) however have indicated possible errors in the various methods used by these authors to determine molar combining ratios. In some cases, enhancement of the protease activity against ester substrates in the presence of alpha-2 macroglobulin has been reported (Szewczuk and Szczeklik, 1973; Twumazi, et al., 1977).

More recently, Topping and Seilman (1979) have shown that alpha-2 macroglobulin appears to bind trypsin in two kinetically differentiated modes, and that by mathematical extrapolation, up to 16 molecules of trypsin may be bound to a single alpha-2 macroglobulin molecule under suitable conditions (Topping and Craven, 1979).

(ii) Molecular Microheterogeneity of Alpha-2 Macroglobulin

Genetic variants of human alpha-2 macroglobulin have been reported only in a population of South American Indians (Gallango and Castillo, 1974). However, electrophoretic molecular heterogeneity of alpha-2 macroglobulin was described by Ganrot and Laurell (1966) and later by Saunders, et al., (1971) whose data indicated that the macroglobulin undergoes changes in both shape and charge on interaction with the protease, which is reflected in a change in electrophoretic mobility.

Isoelectric microheterogenieity of alpha-2 macroglobulin, within a limited acidic pH range, has been reported by Frenoy and Bourillon (1974) who demonstrated differing sialic acid and galactose contents of the four isoelectric variants. However more recently Ohlsson and Skude (1976) have identified two variants of isoelectric points (pI) 6.0 and 5.1 which, in the presence of excess protease, reverted to the single pI 6.0 component.

(iii) Interaction between alpha-1 antitrypsin and alpha-2 macroglobulin in the elimination of circulating proteases

Ohlsson (1975) reviewed the experimental data on the interaction of the two major antiproteases in the dog, which demonstrated both the preferential binding of

radiolabelled trypsin to alpha-2 macroglobulin, and the transfer of radiolabelled trypsin from infused alpha-1 antitrypsin to alpha-2 macroglobulin and its subsequent recovery from the reticulo-endothelial system. Similar results have been reported after infusing radiolabelled Bacillus subtilis endopeptidases into rabbits (Debanne, Regoeczi and Dolovich, 1973). More recently Topping and Seilman (1979) have shown that alpha-2 macroglobulin is the primary protease inhibitor in human serum and only when virtually all its binding capability has been used up does alpha-1 antitrypsin contribute to the binding pattern.

5.3.3. INTERALPHA-TRYPSIN-INHIBITOR

The interalpha-trypsin inhibitor of human plasma was identified and partially characterised by Heide, Heimburger and Haupt (1965). Recently, Barnett, Gottovi and Johnson (1975) reported a significantly low mean serum interalpha trypsin inhibitor concentration in a series of patients with chronic obstructive pulmonary disease. These authors suggested this may be associated with low levels of a mucus specific, bronchial antiprotease which may be an interalpha trypsin inhibitor breakdown product (Hochstrasser, Reichart and Heimburger, 1973). The significance of this suggestion is at present unclear.

5.4. SERUM ANTIPROTEASES IN THE HORSE

5.4.1. BIOCHEMISTRY

Korshum (1902) identified two physicochemically distinct rennin inhibitory components after salt precipitation of horse serum. Nakamura and Wakeyama (1961) later identified two electrophoretically distinct antiprotease fractions in horse serum, migrating at pH 8.6 in the albumin and alpha-2 zones respectively. By combining paper electrophoresis, pH 6.2, with a semi-quantitative casein precipitation inhibition test, Fossum (1970) showed the electrophoretically fast and slow inhibitors to be equally active against both trypsin and chymotrypsin. Later, Nakamura, Ogata and Suzuno (1972) measured the trypsin and chymotrypsin inhibitory activity of eluted paper electrophoretic fractions using an ester substrate and concluded that the albumin zone component inhibited both trypsin and chymotrypsin, while the quantitatively less significant alpha-2 component inhibited only trypsin.

This biphasic distribution of horse serum antiprotease activity was confirmed using fibrinagar electrophoresis by Erickson (1975) and later by Von Fellenberg (1978a). Von Fellenberg (1978b) identified electrophoretic heterogeneity of the albumin zone inhibitor and suggested that, by analogy with other species, the alpha-2 inhibitor was probably alpha-2 macroglobulin. This author also established that while the alpha-2

inhibitor is active against the proteolytic activity of trypsin, chymotrypsin, elastase and plasmin, the albumin inhibitor is active against only trypsin, chymotrypsin and elastase. This data supports the earlier results of Fossum (1970) who demonstrated a relatively wider range of antiprotease specificity associated with the electrophoretically faster inhibitor. Von Fellenberg (1978b) quantitated the antiprotease activity of the alpha-2 inhibitor by densitometric scanning of the stained fibrinagar electrophoretogram, reporting the mean percentages of the total serum anti-trypsin, antichymotrypsin and antielastase activity to be 45 $\pm$ 2.7, 53.3 $\pm$ 5.2 and 34 $\pm$ 5.6 respectively.

5.4.2. SERUM TRYPSIN INHIBITORY CAPACITY (STIC) IN THE HORSE

In a comparison of the STIC of a number of species, Ihrig, Kleinerman and Rynbrandt (1971), using the ester substrate method of Eriksson (1965), reported the STIC of the one horse and one pony included in the study to be 1.5 mg/ml and 1.05 mg/ml respectively. Using the same method, Corbella, Ottonello and Ubaldi (1977) reported the mean STIC of eight clinically normal horses to be 1.046 $\pm$ 0.49 mg/ml. Corbella (1975) had earlier published some STIC values of groups of clinically normal and diseased horses, however the poor presentation of their results makes them uninterpretable.

Breeze et al., (1977), using the Eriksson (1965) method without the addition of calcium to the substrate buffer, established a mean STIC of 1.61 $\pm$ 0.30 mg/ml for 46 respiratory disease free animals. The descrepancy between these results and those of Corbella, et al., (1977) may, in part, be due to the apparent stabilising effect of calcium ions on trypsin (Troyer and Moskowitz, 1968).

5.4.3. ELECTROPHORETIC DETERMINATION OF SERUM ANTITRYPSIN ACTIVITY IN THE HORSE

Although the albumin zone mobility of the electrophoretically faster antiprotease was well documented the literature, Corbella et al., (1977), Breeze et al., (1977) and Littlejohn (1978) determined the alpha-1 or total alpha globulin concentration as part of an investigation of the association of serum antiprotease activity and chronic pulmonary disease in the horse. The former were unable to establish a correlation of alpha-1 globulin concentration and STIC, and all groups considered the parameter to be of little value in their investigation.

Prior to electrophoresis, Corbella et al., (1977) treated control horse serum with 30% acetone (pH 5.0) which resulted in the removal of the total serum globulins and indicated a point of differentiation of the albumin and alpha-1 globulin fractions in the test sera. However the published data of Eriksson

(1965) shows that 30% acetone treatment of human serum resulted in the selective removal of only the alpha-1 antitrypsin and the C'3 bands, the remaining globulins being unaffected. Corbella, et al., (1977) also examined the antiprotease activity of a number of sera by immunoelectrophoresis against anti-whole horse serum, basing their interpretation of the results upon the data of Rouse (1971) who had identified only the IgG, IgM and IgG(T) precipitin arcs. These authors went on to identify the major alpha and beta zone precipitin arcs as alpha-1 antitrypsin and transferrin respectively. However, assuming their empirical identification of the transferrin arc is correct, then the alpha zone arc is most probably alpha-2 macroglobulin (Lavergne and Raynaud, 1970).

The specific antiprotease components of horse serum have not as yet been identified and physicochemically characterised. However, Braend (1967, 1970) has suggested that, on the basis of both his own unpublished data, and by analogy with the antiprotease in man, some of the polymorphic protein zones migrating anodally to albumin after acidic starch gel electrophoresis may represent the equine homologue of human alpha-1 antitrypsin.

5.5. SERUM PROTEIN POLYMORPHISM IN THE HORSE

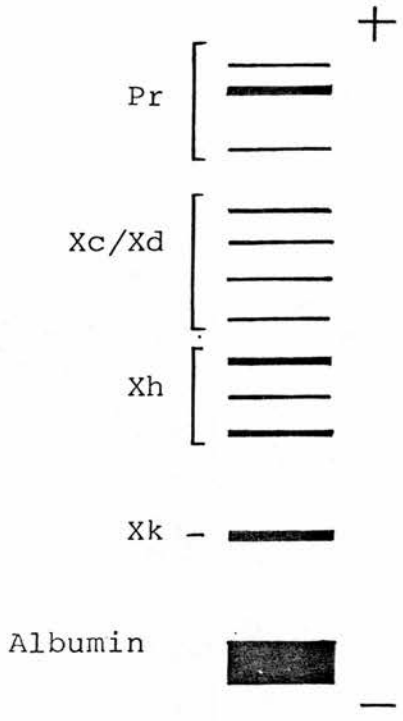
5.5.1. GENERAL

The genetically determined electrophoretic heterogeneity of a number of horse serum proteins is well established, and has been reviewed by Braend (1972) and Sandberg (1974). Serum protein polymorphism in the horse is used primarily in the definitive identification of presumed families (Scott, 1976), or in the investigation of the phylogenetic relationship between various breeds of equidae (Blokhuis and Buis, 1979).

5.5.2. ACIDIC PREALBUMINS

After starch gel electrophoresis (pH 5.0), a large number of acidic prealbumin protein zones are apparent (Fig. 5.1) (Gahne, 1966; Braend, 1967). The most anodal group of bands are the allele products of the highly polymorphic Pr (prealbumin) locus (Gahne, 1966; Braend, 1970), and the most cathodal are the products of the triallelic Xk locus (Braend, 1967). Anodal to the Xk protein, in order of increasing electrophoretic mobility lie a discrete group of bands, tentatively designated the Xh protein (Braend, 1967), and an indefinite zone tentatively considered to contain two variable and overlapping heterogeneous protein systems, Xd and Xc (Braend, 1967).

Fig. 5.1. Schematic diagram of horse prealbumins after acidic starch gel electrophoresis (pH 4.8).



The Pr locus is defined by ten known autosomal codominant alleles (Braend, 1970; Scott, 1976; Scott, 1977) and is potentially the single most effective locus in paternity exclusion tests (Scott, 1976). However difficulties involved in the running and interpretation of acidic starch gels (Scott, 1976) are reflected in the results of recent Horse Blood Typing Comparison Tests (Buis, 1977, pers. comm.; Storset and Braend, 1979, pers. comm.) which show marked disagreement between those laboratories involved in phenotyping the Pr protein. The physiological function of the acidic prealbumins has not been investigated, although

Buis (1976) stated, without evidence, that the Pr protein is a thyroid hormone carrier protein.

Using a specific chromatic stain Gahne (1966) and Scott (1972) demonstrated multiple esterase zones in horse serum after acidic starch gel electrophoresis (pH 4.0 - pH 5.4). The heterogeneity of these prealbumin esterase zones (Es) is defined by at least seven autosomal codominant alleles (Gahne, 1966; Scott, 1972; Fisher and Scott, 1978), and a single recessive null allele (Gahne, 1966; Kaminski 1978). On starch or polyacrylamide gel electrophoresis at pH 8.9, these esterases migrate in the fast alpha-1 region (Gahne 1966; Juneja, Gahne and Sandberg, 1978) although the relative electrophoretic mobility of the allele products appears pH dependant (Gahne 1966; Kaminski, 1974). The function of these serum esterases is also unknown.

5.5.3. OTHER POLYMORPHIC PROTEIN SYSTEMS

Genetically determined molecular heterogeneity has been demonstrated in a number of serum proteins in the horse, i.e. albumin (Braend and Efremov, 1965), transferrin (Braend and Stormont, 1964), Gc-vitamin D binding protein (Juneja, Gahne and Sandberg, 1978) and haemoglobin (Braend and Stormont, 1964). Recently Trommershausen-Smith and Suzuki (1978b) showed that the acidic prealbumin Xk is identical to a polymorphic postalbumin (Pa) locus described by Suzuki and Stormont (cited by Trommershausen-Smith and Suzuki, 1978b).

Genetically determined variation in plasma pseudocholinesterase activity was described by Gahne, Bengtsson and Rendel (1970). The pseudocholinesterases of horse serum were originally described by Augustinsson (1961) and were shown by Kaminski and Gajos (1964) and Gahne (1966) to appear in the slow alpha-2 region after starch gel electrophoresis (pH 8.6 - 8.9).

5.6. SERUM ANTIPROTEASES IN OTHER SPECIES

At least two electrophoretically, quantitatively and functionally distinct serum antiproteases have been identified in the alpha-1 and alpha-2 regions of the guinea pig, hamster, rabbit, pig, sheep, cow, goat and dog (Nakamura and Wakeyama, 1961; Fossum, 1970; Nakamura, et al., 1972). A comparative study of the biochemically determined STIC in a number of species (Ihrig, et al., 1971) has shown that most have values within the range 1 mg/ml to 2 mg/ml although the hamster and guinea pig have significantly lower and higher levels respectively.

An alpha-1 antiprotease has been partially physicochemically characterised in sheep serum (Martin, 1961) and in bovine serum (Tan and Gans, 1972). In the dog, Abrams, Kimbel and Weinhaum (1978) have recently isolated and characterised two isomeric forms of alpha-1 antiprotease, and in the rabbit, Koj, et al., (1978) demonstrated electrophoretic and isoelectric hetero-

geneity of an alpha-1 antitrypsin. Two electrophoretically and functionally distinct alpha-1 antiproteases, have been identified in the guinea-pig (Kobayashi and Nagasawa, 1974). In the mouse (Myerowitz et al., 1972) and rat (Rosenberg, Roegner and Becker, 1976) two immunologically distinct alpha-1 antiproteases have been isolated.

Kueppers and Ganesan (1977) described genetic polymorphism of an alpha-1 antitrypsin homologue in non-human primate serum. However, there appear to be no reports describing such genetic polymorphism in non-primate species.

The alpha-2 antiprotease has been identified as alpha-2 macroglobulin in the dog (Ohlsson, 1971) the rabbit (Berthillier, Got and Bertagnalio, 1968) and the pig (Baumstark, 1973).

5.7. AN ANIMAL MODEL OF ALPHA-1 ANTITRYPSIN DEFICIENCY: ROUND HEART DISEASE OF TURKEYS

Meirom et al., (1974) described hypoproteinaemia and alpha globulin deficiency in turkeys affected with round heart disease, a condition pathologically characterised by cardiac hypertrophy and P.A.S. positive, diastase resistant, intracytoplasmic globules in the hepatocytes, (Neumann et al., 1976). These globules contain material immunologically cross reactive with serum alpha globulin (Neumann et al., 1976). The

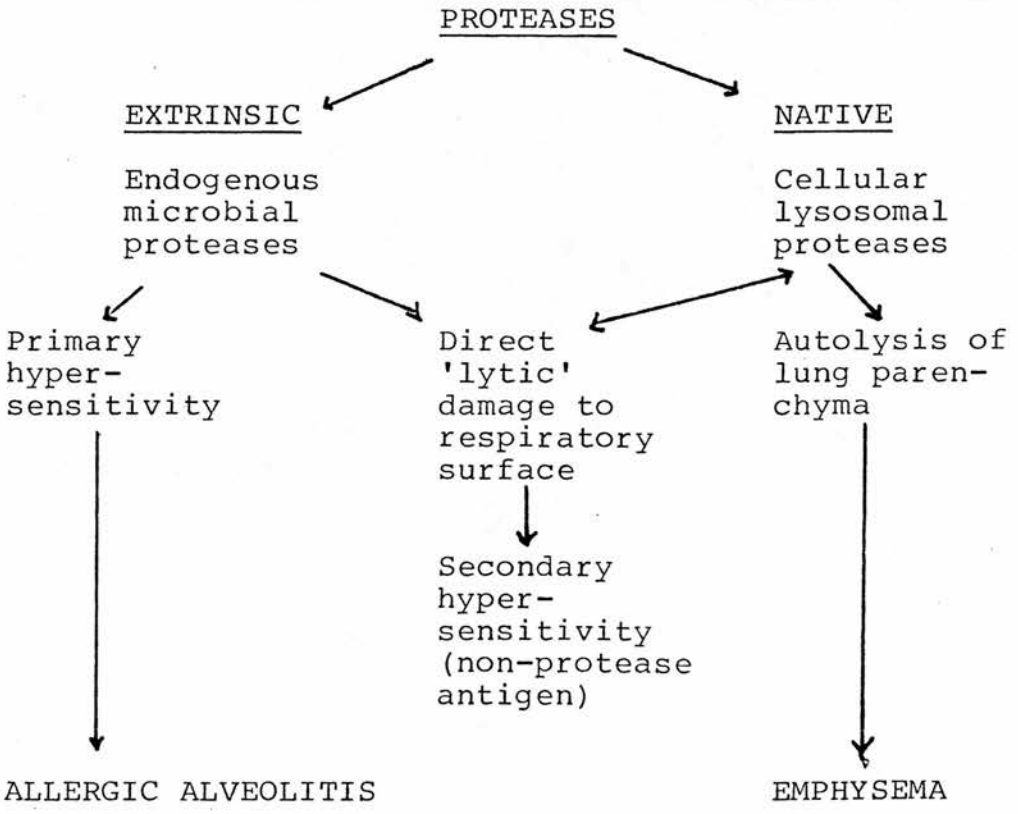
staining intensity of those bands presumed to represent an alpha antiprotease after acid starch gel electrophoresis is markedly diminished in serum from affected birds (Martin et al., 1976).

5.8. PATHOPHYSIOLOGY OF PROTEASES IN PULMONARY DISEASE

Figure 5.2 summarises the possible pathophysiological mechanisms of pulmonary damage by protease enzymes.

Fig. 5.2

Possible pathways of pulmonary damage by proteases.



In man, Kueppers and Bearn (1966b) described the 'in vitro' inhibition of human leucoproteases by alpha-1 antitrypsin, and postulated that a dysproteinaemia of the inhibitor may result in uncontrolled, native leucoprotease mediated lysis of pulmonary parenchyma and consequent emphysema.

In contrast to antiprotease deficiency associated chronic pulmonary disease in man, C.O.P.D. in the horse is an apparent local hypersensitivity to fungal antigens (1.1.), and the mechanisms mediated by extrinsic proteases may be of greater importance. However, in man there are no reports of a direct association of antiprotease deficiency with the development of respiratory hypersensitivity, although reports of the nature of fungal antigens suggest that surface active pulmonary antiproteolysis may play a role in defence against the development of the disease (1.1.).

5.8.1. NATIVE PROTEASES AND PULMONARY DISEASE

(i) Animal Models of Protease-Mediated Chronic Pulmonary Disease.

Emphysema has been induced in rats (Gross et al., 1964) and rabbits (Caldwell, 1972) after endotracheal instillation of solubilised papain, and Goldring et al., (1972) induced severe parenchymal destruction and emphysema in hamsters after intratracheal aerosolisation of a range of proteases. Emphysema has been successfully induced in dogs by intratracheal instillation

of both human and canine leucocyte homogenate (Marco et al., 1971; Kimbel et al., 1972) and canine alveolar macrophage homogenate (Kimbel et al., 1972), although Kimbel et al., (1972) and Kimbel and Weinbaum (1975) showed that only cell homogenates with significant proteolytic activity at neutral or slightly alkaline pH were able to induce emphysema.

(ii) Alkaline Lysosomal Proteases of Granular Leucocytes.

Potentially autolytic native granulocyte proteases in the lung may arise from effete leucocytes which are normally sequestered in the mammalian pulmonary microcirculation (Bierman, Kelly and Cordes, 1955; Heineman and Fishman, 1969). However, chronic low grade respiratory tract inflammation will result in persistant localised exposure to proteases arising from disintegrated inflammatory leucocytes.

(a) Elastase-like Esterase: The alkaline fraction of human granulocyte homogenate (Janoff and Scherer, 1968; Janoff and Basch, 1971), has a marked 'in vitro' activity against glomerular vascular basement membranes and canine aortic elastin (Janoff, 1970). It induces a severe localised vasculitis in mice (Janoff, 1970) and causes marked 'in vitro' destruction of human ciliated columnar epithelium (Tegner, 1977). The potency of leucocyte homogenates in inducing experimental emphysema in dogs is directly related to its elastase activity

(Kimbel and Weinbaum, 1975), and rabbit leucocyte homogenate, with no demonstrable elastolytic activity (Janoff, 1972c), failed to induce emphysema in dogs (Kimbel et al., 1972).

Human granulocytic elastases are electrophoretically heterogeneous serine proteases, inhibited by both alpha-1 antitrypsin and alpha-2 macroglobulin (Ohlsson and Olsson, 1974a; 1974b; Schmidt and Haremann, 1974).

Horse leucocytes have been reported to contain three neutral serine elastases (Koj, et al., 1972; Dublin, Koj and Chudzik, 1976), inhibited both by horse serum and human serum (Koj, et al., 1970; 1972).

(b) Collagenase: Human granulocyte collagenases (Lazarus et al., 1968) has only limited activity against glomerular vascular basement membrane (Janoff, 1970). Granulocyte collagenases are inhibited both by alpha-1 antitrypsin and alpha-2 macroglobulin (Ohlsson and Olsson, 1977).

(c) Chymotrypsin-like proteases: Electrophoretically heterogeneous human granulocyte serine proteases which are inhibited by alpha-1 antichymotrypsin but not by alpha-1 antitrypsin have been described by Schmidt and Haremann (1974).

(ii) Alveolar Macrophage Proteases

Human alveolar macrophage homogenate, although a

potent inducer of experimental emphysema in mice (Janoff, 1972b), possesses only weak neutral elastase activity (Janoff, Rosenberg and Galdston, 1971) which is unaffected by alpha-1 antitrypsin (Rosenberg, Sandhaus and Janoff, 1972), and Janoff, et al., (1971) have speculated upon its possible importance in the development of emphysema in the antiprotease deficient individual.

Cohen (1973b) demonstrated an acidic cathepsin-like protease, inhibited by alpha-1 antitrypsin, in human alveolar macrophages, and Cohen and Cline (1971) earlier demonstrated an acidosis within actively phagocytosing alveolar macrophages which would favour the proteolytic activity of this acidic enzyme.

Janoff (1972c) proposed that alveolar macrophages may release their proteases due to exposure to cytotoxic stimuli, and because of their lack of inhibition by alpha-1 antitrypsin these proteases may be involved in the development of emphysema in response to pulmonary irritation in the absence of antiprotease deficiency. This hypothesis is supported by the data of Harris et al., (1975), who demonstrated an increased elastolytic activity in alveolar macrophages of smokers as opposed to non-smokers.

(iv) Local Antiproteolysis in the Respiratory Tract

Alpha-1 antichymotrypsin, alpha-2 macroglobulin and alpha-1 antitrypsin have all been found in normal human

bronchial secretions (Laine and Hagen, 1976), the latter being invariably found, though in reduced amounts, in bronchial washings from PiZZ individuals (Laine, Lebas and Hagen, 1975).

A polyvalent low molecular weight, mucus specific inhibitor has been demonstrated in human bronchial secretions, forming 70 percent of the total protease inhibitory activity (Reichart, Hochstrasser and Conradi, 1972). This inhibitor which occurs in both a free and protease-complexed form was reported to be antigenically cross reactive with the interalpha trypsin inhibitor of human serum by Hochstrasser, et al., (1975), although this was not confirmed by Laine and Hagen (1976). This mucus specific inhibitor has been shown to be a potent inhibitor of trypsin and chymotrypsin (Reichart, et al., 1972), granulocyte elastase but not granulocyte collagenase (Ohlsson and Tegner, 1976).

In man, alpha-1 antitrypsin has been demonstrated in the alveolar lumen and the interalveolar septae, both free and in macrophage cytoplasm (Cohen, 1973b). This alpha-1 antitrypsin probably arises from serum alpha-1 antitrypsin which crosses into the alveolus in the form of an unstable complex with IgA and which subsequently dissociates in contact with exocrine secretions (Tomasi and Hauptman, 1974).

An alpha-1 trypsin inhibitor has been identified in canine lung surfactant protein (Tuttle and Westerberg, 1974), and more recently a lung specific antiprotease,

able to prevent experimentally induced emphysema, has been identified in alveolar washings from canine lungs. (Weinbaum, et al., 1976).

Von Fellenberg (1978a) identified two tissue specific antiproteases in bovine lung homogenate, but was unable to demonstrate tissue specific antiprotease activity in equine lung homogenate.

5.8.2. EXTRINSIC PROTEASES AND PULMONARY DISEASE

Endogenous antigenic proteases are produced by a number of micro-organisms (Matsubara and Feder, 1971) and vary in susceptibility to human plasma protease inhibitors (Zuber, 1975). Of probable importance in pulmonary disease in man are the serine endopeptidase of Bacillus subtilis and Aspergillus species, (Zuber, 1975), and the chymotrypsin-like proteases of the Thermoactinomycete species (Nicolet and Bannerman, 1975; Roberts, 1978). Human alpha-1 antitrypsin and alpha-2 macroglobulin inhibit the endopeptidases of B. subtitis (Wicher and Dolovich, 1973) and Asp. Oryzae (Fossum, 1970), and alpha-1 antitrypsin variably inhibits the proteases of some Thermoactinomycete species (Roberts, 1978).

Respiratory hypersensitivity has been reported in man following exposure to trypsin (Pilat and Teculescu, 1975; Colten et al., 1975), and Pepys et al., (1969) reported respiratory hypersensitivity to B. subtitis enzyme extracts amongst a non-atopic population exposed to the aerosolised enzyme. Slavin (1978) however failed

to demonstrate a hypersensitivity component in a group of similarly exposed, mainly non-atopic patients showing signs of chronic pulmonary disease.

Allergic bronchopulmonary aspergillosis is a disease of atopic individuals (Pepys, 1969) involving both humoral and cellular hypersensitivity mechanisms (Forman et al., 1978), and pulmonary aspergilloma, a focal mycosis affecting mainly non-atopic individuals, has been shown to involve both humoral and cellular hypersensitivity mechanisms (Forman et al., 1978). Biquet and Vernes (1974) have indicated that antigenic proteases released by actively growing fungal mycelia may result in both hypersensitization of the host and direct lysis of pulmonary tissue.

Extrinsic allergic alveolitis or hypersensitivity pneumonitis is a disease of non-atopic individuals (Pepys, 1969) associated with exposure to a number of inhaled antigens, including those of Micropolyspora faeni and other Thermoactinomycetes (Biquet et al., 1974). The role of direct lysis of the respiratory surfaces by protease antigens in the pathogenesis of the disease is unclear (Nicolet, Bannerman and de Haller, 1975). However the basic immunopathogenesis appears to involve hypersensitization of the host to endogenous fungal antigens (Schatz, et al., 1977; Moore, 1978). In the horse, inhalation exposure to fungal antigens, particularly those of

M. faeni is important in the development of C.O.P.D. (McPherson et al., 1979a), although the specific importance of proteolytic antigens has not been established.

The role of intracellular alpha-1 antitrypsin in human alveolar macrophages (Cohen 1973b) in the defence against inhaled protease antigen is unclear. M. faeni activates complement independantly of its protease activity (Marx, 1978), and although C'3 is a potent macrophage activator (Allison, 1978) human alveolar macrophages are relatively inactive against M. faeni.

5.8.3. ALPHA-1 ANTITRYPSIN DEFICIENCY AND ALLERGIC LUNG DISEASE:

A number of authors have investigated the association of asthma and the subsequent development of emphysema with alpha-1 antitrypsin deficiency. Szczeklik et al., (1974) reported a significantly increased incidence of emphysema in asthmatics with low serum antiprotease levels, although the results of Altay, et al., (1973), Katz, Lieberman and Seigel (1976) and Schwartz et al., (1977) fail to show an increased incidence of Pi deficient phenotypes in atopic asthmatic populations. Katz et al., (1976) however did report an association of the PiZZ phenotype with increased severity of the asthma. Arnaud et al., (1976) found a significantly increased frequency of deficient Pi phenotypes in a population of non-atopic infant asthmatics, and postulated that in these individuals low serum antiprotease levels may be the triggering factor in the

disease.

5.9. CONCLUSIONS FROM THE LITERATURE SURVEY

Although a considerable amount is known about the molecular properties of human serum antiproteases and the probable role of alpha-1 antitrypsin deficiency in the onset of chronic pulmonary dysfunction, very little is known about the antiprotease activity of horse serum. However a number of basic differences in serum antiproteolysis between man and the horse have been described. Prior to the investigation of the role of antiprotease deficiency as an intrinsic determinant in the onset of equine C.O.P.D., these differences must be more fully elucidated by quantitative and genetic studies of the individual antiproteases in horse serum.

However, major aetiological, pathological and clinical differences exist between antiprotease deficiency associated chronic lung disease in man (Table 5.2) and C.O.P.D. of horses (1.1). Nevertheless the possible role of endogeneous microbial proteases in the onset of hypersensitive lung disease and the reported absence of a lung specific protease inhibitor in the horse suggests that in this species serum antiproteases may play a role in defence of the host against aerogenous sensitization by the fungal antigens incriminated in C.O.P.D.

CHAPTER 6

IDENTIFICATION AND CHARACTERISATION OF AN ALPHA-1 ANTITRYPSIN HOMOLOGUE IN HORSE SERUM

6.1. INTRODUCTION

A homologue of human alpha-1 antitrypsin has not been described in horse serum. Braend (1970) however has suggested that by analogy with the electrophoretic behaviour of human alpha-1 antitrypsin, some of the prealbumin proteins of horse serum after acidic starch gel electrophoresis may correspond to the human antiprotease. This proposition is supported by the marked similarity in isoelectric point and molecular weight of the alpha-1 antiprotease components in the serum of man and a number of other species (Table 6.1). In this chapter, an antiprotease component in horse serum with albumin zone mobility after electrophoresis at alkaline pH was identified and shown to be associated with a polymorphic acidic prealbumin protein.

The chapter is divided into 4 parts:-

Part I. Investigation of the electrophoretic distribution of the antiprotease activity in horse serum at alkaline pH.

Part 2. The technique of acidic starch gel electrophoresis (ASGE) and identification of the major prealbumin proteins in horse serum after ASGE, including the probable homologue of human alpha-1 antitrypsin.

Part 3. Examination of the polymorphic nature of the alpha-1 antitrypsin homologue using three different methods,

(i) Acidic starch gel electrophoresis.

(ii) Isoelectric focusing.

(iii) Immunofixation electrophoresis.

Part 4. Discussion of some of the experimental results in relation to those of other workers in the field.

TABLE 6.1.

ISOELECTRIC POINTS (pI) AND MOLECULAR WEIGHTS (MW) OF THE ALPHA-1 ZONE PROTEASE INHIBITORS IDENTIFIED IN THE SERUM OF A NUMBER OF SPECIES.

SPECIES	pI	MW	REFERENCE
Human	4.85-4.95	52-53,000	Saklatvala, et al., (1976)
Dog	4.40-4.52	58,000	Abrams, et al., (1978)
Rat	-	52-62,000	Rosenberg, et al.,(1976)
Mouse	4.6 - 4.7	58,000	Myerowitz, et al.,(1972)
Guinea Pig	-	78,000	Kobayashi & Nagasawa (1974)
Sheep	4.3	-	Martin (1961)
Rabbit	4.8 - 5.0	58,000	Koj, et al., (1978).

PART I

6.2. THE ELECTROPHORETIC DISTRIBUTION OF HORSE SERUM ANTIPROTEASE ACTIVITY.

6.2.1. INTRODUCTION

Fossum (1970) and Erickson (1975) demonstrated a biphasic distribution of horse serum antiprotease activity about the albumin and alpha-2 regions after zone electrophoresis at alkaline pH. To confirm these observations, the antitrypsin and antichymotrypsin activity of horse serum was determined after electrophoresis on fibrinagar (pH 8.2).

6.2.2. MATERIALS AND METHODS

Discontinuous fibrinagar electrophoresis was carried out using a modification of the method described by Heimburger and Schwick (1962).

(i) Fibrinagar Gel

To 24 ml of 1.5% ($^{w}/v$) suspension of agar[1] in 0.05M barbital buffer (pH 8.2) was added 2 ml of 0.1M calcium lactate and 4ml of 0.4% ($^{w}/v$) bovine fibrin[2] in barbital buffer.

(ii) Electrode Buffer

0.065M barbital-calcium lactate (pH 8.6) (Hirschfield, 1960).

(iii) Electrophoresis

The heated fibrinagar was poured onto 3 defatted microscope slides and after heating at 80°C for 60 minutes in a moist chamber was stored overnight at

1. Ionagar; Oxoid Ltd., Basingstoke, England.
2. Sigma Chemical Co., Poole, England.

4°C prior to use.

Two symmetrical wells were cut on each slide and 5µl horse serum was applied to each well. Using a bromophenol blue dye albumin marker, electrophoresis was carried out at $6Vcm^{-2}$ until albumin had migrated 2.5cm. Central troughs were cut in the slides and approximately 0.1ml of a 0.032% ($^{w}/v$) solution of bovine pancreatic trypsin[1] in 0.0025N HCl was applied to each trough. The gels were incubated overnight in a moist chamber at 37°C.

The procedure was repeated using 0.032% ($^{w}/v$) bovine chymotrypsin[1] in 0.0025N HCl.

6.2.3. RESULTS

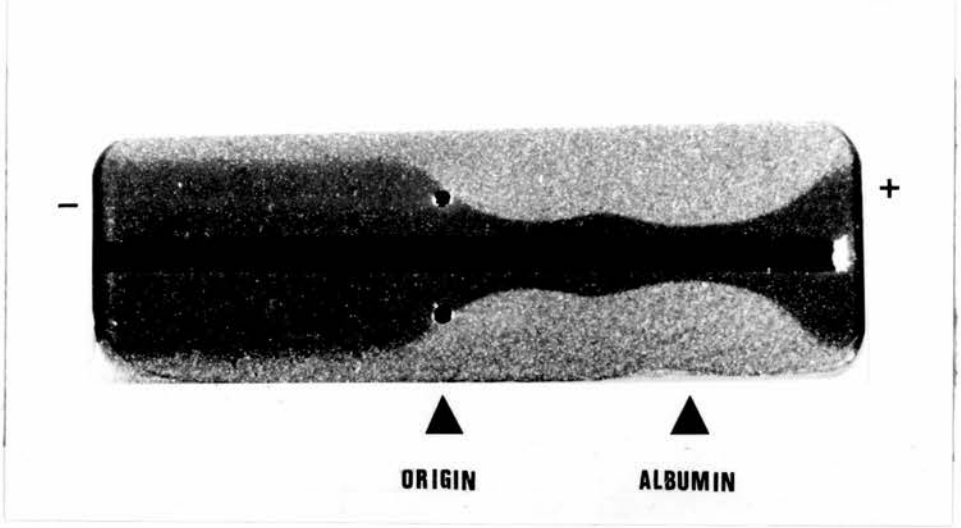
Serum antitrypsin and antichymotrypsin activity shows a distinctly biphasic distribution about the albumin and alpha-2 beta zones respectively (Fig. 6.1.). These inhibitory zones show some degree of overlap. This biphasic pattern of antiprotease activity was present in all 6 sera examined.

6.2.4. DISCUSSION

The results of this experiment confirm those of Erickson (1975) and Fossum (1970) showing the occurrence of at least two electrophoretically distinct protease inhibitors in horse serum. The overlapping of the zones demonstrable after fibrinagar electrophoresis may result from radial diffusion of the inhibitors from

1. Sigma Chemical Co., Poole, England.

Fig. 6.1.



Fibrinagar electrophoresis of horse serum. The trough contains 0.032% ($^{w}/v$) bovine trypsin in 0.0025N HCl.

their post-electrophoretic location in the gel, or may indicate an intermediate zone of antiprotease activity. In this latter respect, Von Fellenberg (1978b) has demonstrated up to 4 discrete zones of antitrypsin and antichymotrypsin activity in the albumin, alpha-1 and alpha-2 zones of horse serum after fibrinagar electrophoresis (pH 8.6). However, it was not established by this author whether these represent independant antiprotease species or polymorphic forms of two antiproteases.

PART 2

THE IDENTIFICATION OF AN ACIDIC PREALBUMIN PROTEASE INHIBITOR IN HORSE SERUM.

6.3. ACIDIC STARCH GEL ELECTROPHORESIS (ASGE) OF HORSE SERUM.

6.3.1. INTRODUCTION

ASGE was for some time the basic tool for the identification of genetically determined molecular variants of serum alpha-1 antitrypsin in man (5.3.1.vi). The similarity in molecular properties of the electrophoretically faster antiproteases in human serum and the serum of a number of species (Table 6.1.) prompted the use of this technique in establishing the identity of the albumin zone protease inhibitor in horse serum.

6.3.2. MATERIALS AND METHODS

(i) Serum

Normal horse serum, described in 3.2.2. was used. Care was taken to minimize freeze-thawing of the samples, a procedure shown to result in slight cathodal shifting of some human alpha-1 antitrypsin variants (Hoffmann and Van den Broek, 1977).

(ii) Acidic Starch Gel Electrophoresis

Discontinuous horizontal starch gel electrophoresis was carried out using a modification of the method described by Fagherol (1972b) for the resolution of the electrophoretic variants of human alpha-1 antitrypsin.

(a) Gel Moulds: Moulds (30cm x 12cm x 0.5cm) were constructed from glass strips laid around the periphery of a glass base plate and sealed with starch glue. These moulds were prepared and heated to 90-100°C for 30-60 minutes immediately prior to use.

(b) Gel Buffer Stock Solutions: Two stock solutions were prepared; (A) 5.25g citric acid in 250ml deionised water, (B) 5.75g tris (hydroxymethyl) methylamine (Tris) in 250ml deionised water.

(c) Electrode Buffers: Anode -10g citric acid and 12g tris per litre of deionised water (pH 4.85). Cathode - 37g di-sodium tetraborate and 1g sodium hydroxide per litre of deionised water (pH 9.8). Fresh electrode buffer was made up immediately prior to each run.

(d) Starch: Hydrolysed potato starch[1] was used at a concentration ($^{w}/v$) of 12-14%. Since the degree of hydrolysis of each starch batch varied, the optimum concentration within the above stated range was determined for each new batch used.

(e) Preparation of the Gel: The weighed starch was suspended in 250ml tris-citrate gel buffer in a 2 litre conical flask. The gel buffer (pH 4.3-4.9) was prepared by mixing stock solutions A and B in a predetermined ratio to a volume of 30ml and making up the final volume with deionised water. Initially a

1. Sigma Chemical Co., Poole, England.

1:1 ratio of solutions A and B was used, although increasing the acidic component was found by trial and error to be necessary to achieve optimum resolution of the prealbumin protein zones.

The starch suspension was heated while being continuously agitated until almost boiling, then degassed and poured into the mould. The gel was covered by thin polythene sheeting and a glass plate was laid over the mould. The gel was cooled for 15-30 minutes at room temperature followed by 60 minutes at 4°C.

(f) Sample Application: 1 x 0.5cm inserts of no.17 chromatography paper[1] were soaked in serum and laid within a vertical cut made across the width of the gel 5cm from the cathodal end. Up to 9 inserts were applied to each gel, although duplicate samples were used at the gel edges because of distortion at the gel-mould interface. After placing the inserts, the glass strip along the cathodal width of the gel was detached from the base plate and slid forward to compress the inserts into the gel to ensure good insert-gel contact.

(g) Electrophoresis: The gel was laid longditudinally across the two electrode buffer tanks and the ends were overlapped 2.5cm by conducting bridges dipping into the buffer solutions. This resulted in an electrode gap of 20cm. These bridges

1. Whatman Ltd., Maidstone, England.

were constructed of 3mm chromatography paper[1] sandwiched between single layers of cotton bandage. This arrangement ensured optimum contact with the gel surface and an even electrode gap across the length of the gel, while resulting in minimum electrical resistance. The gel was covered with polythene sheeting during the run to limit evaporation.

Electrophoresis was run initially at 9 Vcm^{-1} (180V) for 30 minutes, after which the inserts were removed and the insert gap closed by sliding forward the cathodal glass strip, taking care to avoid gel distortion. Electrophoresis was continued at 20Vcm^{-1} (400V) until the visible borate boundary zone had migrated 10cm from the insert line. The voltage across the electrode gap was periodically checked by galvanometer. The gel was cooled by placing ice filled perspex tanks over the surface.

(h) Slicing and Staining: The gel edges were separated from the mould using a scalpel blade and the glass strips removed. 3mm thick perspex strips were laid along the length of the gel and thin steel wire was used to slice the gel horizontally. The gel slices were separated by surface adhesion to thick polythene sheeting. Prior to staining each gel was numbered with haemoglobin solution.

Routine protein staining was carried out for 60 seconds in 1% ($^{W}/v$) nigrosine prepared by dissolving

1. Whatman Ltd., Maidstone, Kent.

5g water soluble nigrosine salt in a 5:7.5:1 solution of distilled water / methanol / glacial acetic acid. After washing for 5 minutes in tap water, the gels were destained overnight in 5:5:1 water / methanol / glacial acetic acid solution.

Gels were stored in the destaining solution for up to 6 months.

(i) Recordings: Gel and serum reference numbers.
Starch concentration and batch number.
Gel buffer ratio (A:B).
Electrode buffers.
Running time and voltage.
Staining.

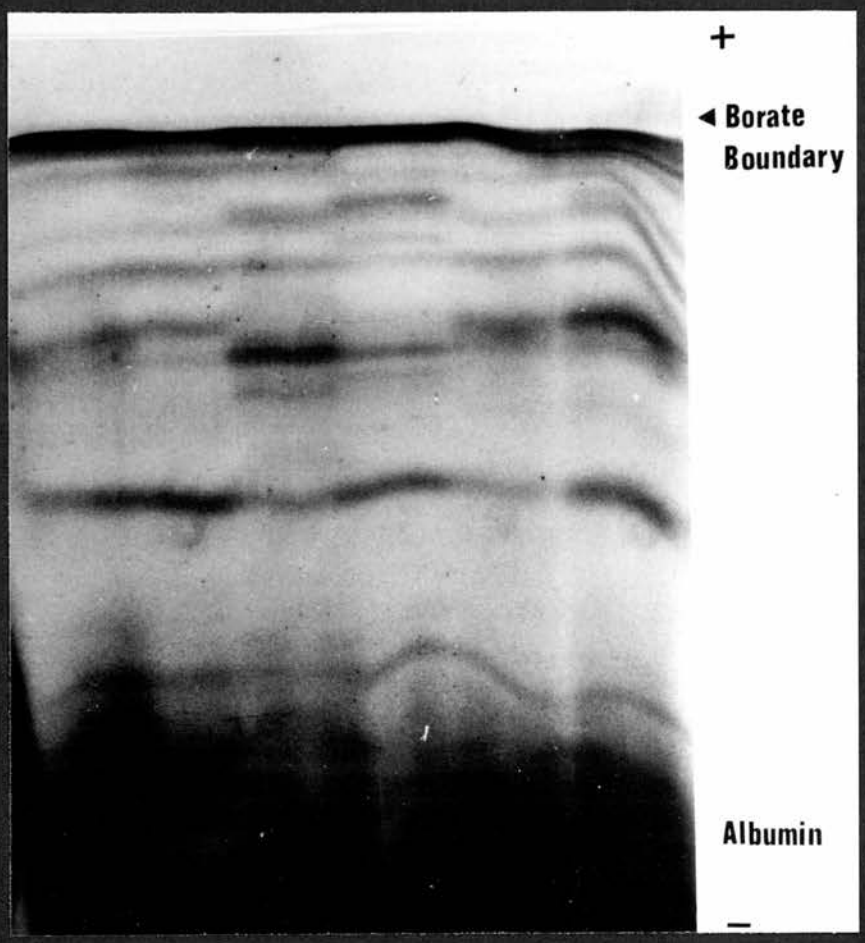
6.3.3. RESULTS

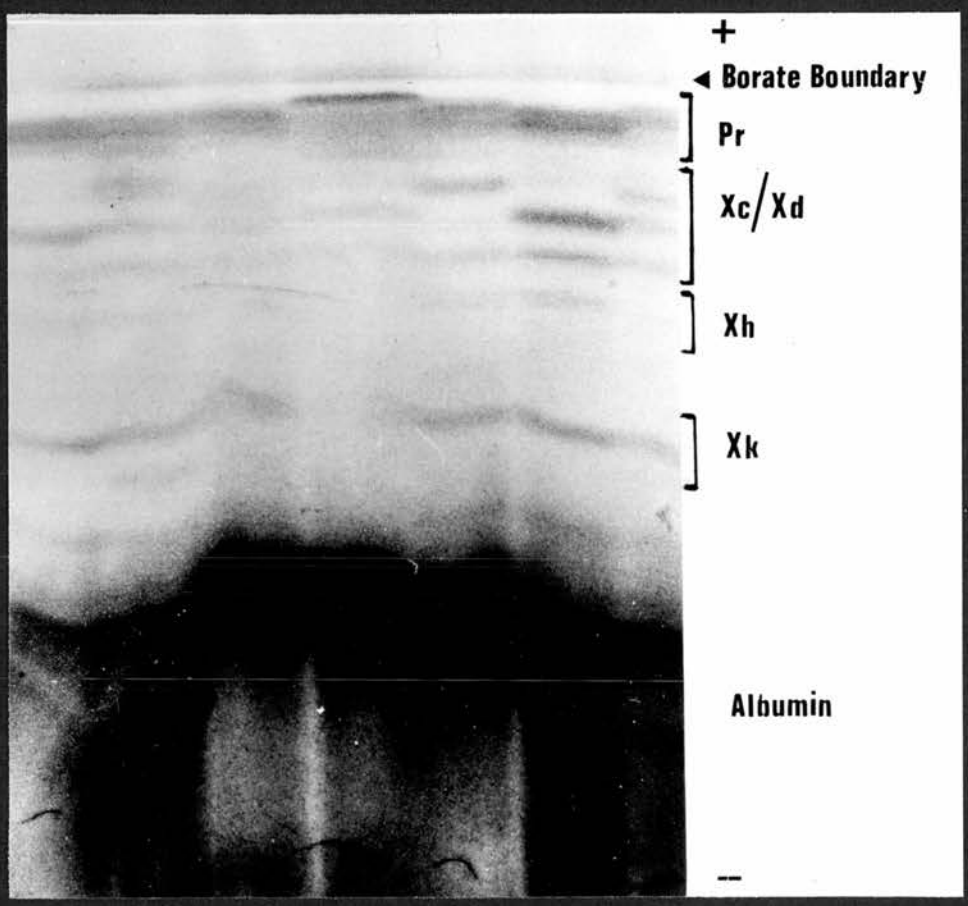
Initially, gel buffer was prepared using equal amounts of stock solutions A and B (pH 4.9). The appearance of the prealbumin protein zones on these gels is shown in Figure 6.2. The more anodal of the prealbumin bands appeared condensed into a homogenous zone immediately cathodal to the borate boundary.

Lowering the gel buffer pH to 4.7 by increasing the acidic component (A) resulted in separation of the most anodal zone into discrete bands which migrated in the region 0.5-1cm cathodal to the borate boundary

Fig. 6.2. Acidic starch gel electrophoresis (pH 4.9) of horse serum.

Fig. 6.3. Acidic starch gel electrophoresis (pH 4.7) of horse serum. The division of the prealbumin zones into the individual systems suggested by Braend (1967) is shown.


+
Borate Boundary
Albumin
–

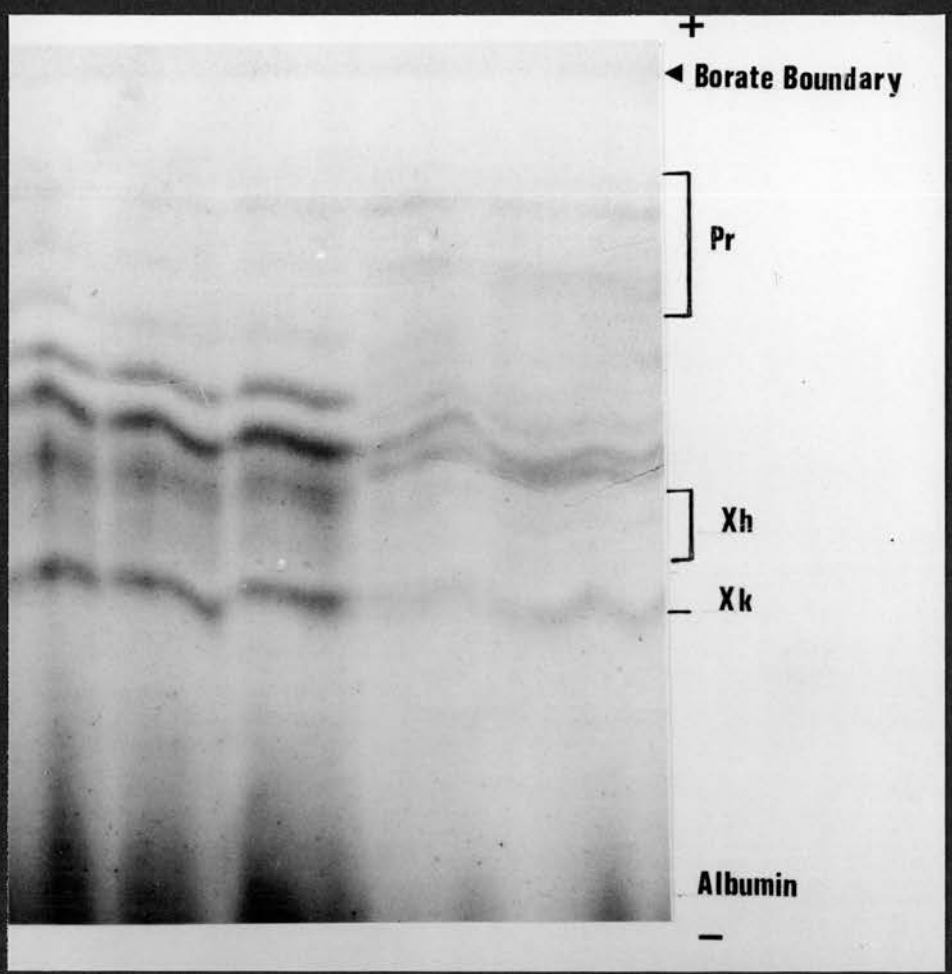

+
Borate Boundary
Pr
Xc/Xd
Xh
Xk
Albumin
–

(Fig. 6.3). However, a single protein band was apparent at the boundary (Fig. 6.3.).

The appearance of the prealbumin protein zones after ASGE (pH 4.7) is similar to that after ASGE (pH 5.0) using a discontinuous tris-citrate/borate buffer system (Braend, 1967). This author tentatively divided these zones into 4 or 5 protein groups or systems. The most anodal and cathodal zones were designated Pr (prealbumin) and Xk respectively (Fig. 6.3.). The latter system was shown to be a genetically determined polymorphic protein, defined by at least 3 autosomal codominant alleles. Between these two protein systems, in order of increasing Rf, Braend tentatively described a discrete Xh system and two overlapping systems designated Xc and Xd (Fig. 6.3.). The X indicated unknown and the lower case letter designated the order of migration.

By further increasing the acidic gel buffer component, reducing the pH to 4.3, the protein bands were pulled further away from the borate boundary (Fig. 6.4.). At this pH the Pr system was separated into a variable number of discrete bands (Fig. 6.4.). The resolution of the acidic prealbumin zones achieved after ASGE (pH 4.3) was comparable to that shown by both Braend (1970) and Trommershausen-Smith and Suzuki (1978a) using a discontinuous tris-citrate/borate system (pH 4.8).

Fig. 6.4. Acidic starch gel electrophoresis (pH 4.3) of horse serum. Two sera are shown and the Xk, Xh and Pr systems described by Braend (1967, 1970) are indicated.


+
Borate Boundary
Pr
Xh
Xk
Albumin
–

Preliminary studies of the effect of starch concentration on the resolution of the prealbumin bands showed that 13% gels resulted in optimum band separation. These gels had good handling characteristics and minimal electrical resistance with consequently less tendency to overheat. Subsequently all gels were prepared at a basic 13% starch concentration with only minor modifications in concentration for each new batch used. The most critical factor in determining the quality of resolution of the prealbumin protein zones was gel buffer pH. Throughout all experiments using ASGE, the gel pH was maintained between 4.3 and 4.52 depending on the starch batch used, the optimum pH being determined for each batch.

6.3.4. DISCUSSION

The ASGE (pH 4.3) technique in this study presented a number of technical difficulties, principally associated with the reproducability of separation and distortion of the protein bands. These difficulties have also been encountered using ASGE for differentiation of the Pr allele products in horse serum (Scott, 1976; Braend, 1979, pers. comm.). To achieve consistent results with the present technique particular care has to be paid to the following details; maintaining constant pH and molarity of the buffer systems, careful closure of the gel following insert

removal and effective cooling of the gel during the high voltage phase of the run. Nevertheless, all gels were repeated to limit misinterpretation of the prealbumin band patterns.

Although the acidic prealbumin protein systems Pr, Xk and the aliesterases (Es) have been extensively studied in relation to their genetically determined polymorphisms (5.5.2.), there is little published data on the number, identity and function of the proteins which make up the complex pattern of prealbumin bands after ASGE. Thus, it was necessary to identify individual prealbumin proteins particularly those with protease inhibitory activity.

6.4. IDENTIFICATION OF PREALBUMIN PROTEINS AFTER ASGE (pH 4.3) OF HORSE SERUM.

6.4.1. DIFFERENTIAL STAINING

(i) Ceruloplasmin

Following ASGE (pH 4.3) of 8 horse sera, the top gel slice was stained with 1% nigrosine while the lower slice was stained for ceruloplasmin by immersion in 0.5% ($^{w}/v$) p-phenylenediamine dihydrochloride in 1M sodium acetate buffer (pH 5.6) for 40 minutes at 37°C.

Ceruloplasmin appeared as a diffuse zone immediately anodal to the insert line and did not overlap the prealbumin zones.

(ii) Lipoproteins

The appearance of lipoproteins in the acidic prealbumin region was investigated by prestaining the serum with 1% (w/v) Acetylated Sudan Black in ethanol using the method of Sargent and George (1975). Lipoprotein appeared immediately anodal to the insert line and did not overlap the prealbumin zones.

6.4.2. HAEMOGLOBIN BINDING

(i) Introduction

The presence of haptoglobin (Hp) in the prealbumin region after ASGE was investigated by incubating serum with fresh horse haemoglobin prior to electrophoresis.

(ii) Materials and Methods

Horse haemoglobin (Hb) was prepared by hypotonic lysis of washed red cells as previously described (3.2.3.(i)) and was added to fresh non-haemolysed serum in a serum:Hb ratio of 9:1. At this ratio serum will be in slight excess (3.3.2(i)). Control sera were diluted 9:1 in O.OlM phosphate buffered saline (PBS) (pH 7.4). After incubation for 30 minutes at room temperature paired samples of the serum-Hb mixtures and serum controls were simultaneously subjected to ASGE at pH 4.9 and pH 4.3. The top gel slice was stained

by immersion in 100ml of 0.2% ($^{v}/v$) benzidine in 0.5% ($^{v}/v$) glacial acetic acid to which was added 0.6ml H_2O_2. The bottom slice was stained with 1% nigrosine.

(iii) Results

Following ASGE at pH 4.9 and 4.3, the Hp-Hb complexes were identified by benzidine staining as forming a diffuse zone extending about 7mm anodally from the insert line. The prealbumin protein zones after ASGE of the serum-Hb incubates and the serum controls at pH 4.9 and 4.3 are shown in Figures 6.5 and 6.6. At pH 4.9 the addition of Hb to sera A and B prior to electrophoresis resulted in the removal of the protein bands tentatively described as the Xh system by Braend (1967) (Fig. 6.5.). At pH 4.3 however, the addition of Hb to sera C, D and E resulted in the removal or loss of staining intensity of bands anodal to and within the so-called Xh system (Fig. 6.6.). In serum C a single Xh band is apparent and is unaffected by Hb, although there is loss of staining intensity of the group of bands immediately anodal to the Xh zone. In serum D the most anodal and cathodal of the three Xh bands disappear after Hb addition. There is also loss of staining intensity of the remaining bands within and anodal to the Xh region, although the overall staining intensity of the prealbumin proteins is diminished. In serum E there is a loss of staining intensity of all three bands within the Xh zone,

Fig. 6.5. ASGE (pH 4.9) of horse sera A and B before and after incubation with haemoglobin (Hb).

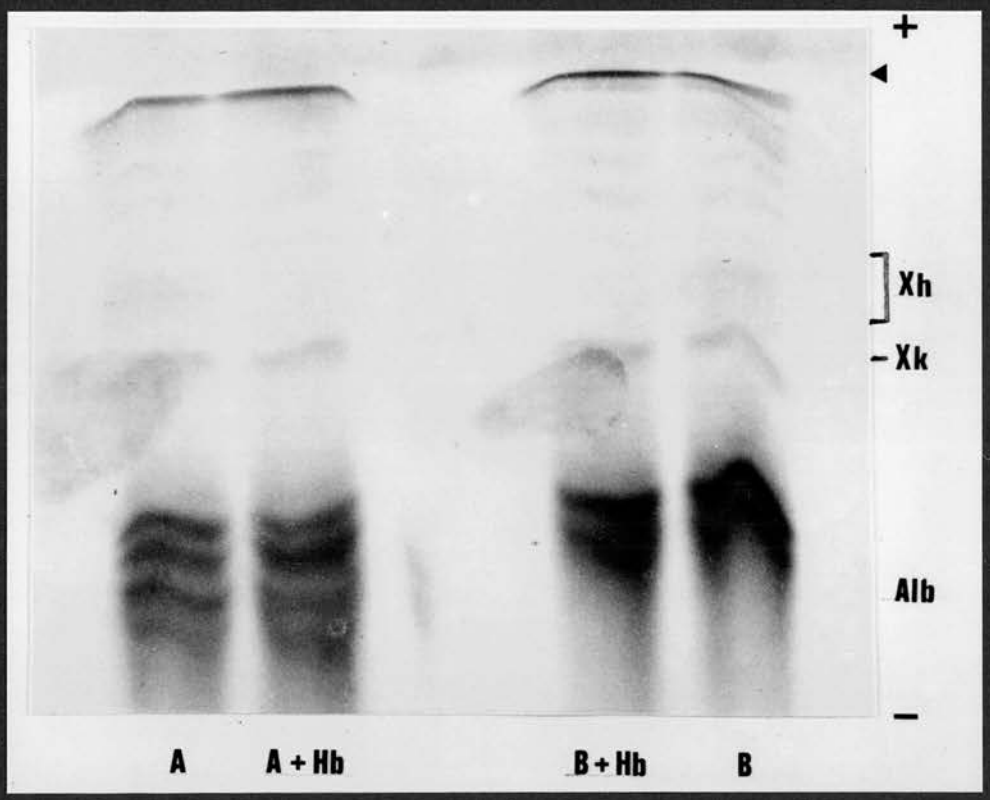
◄ - Indicates borate boundary.

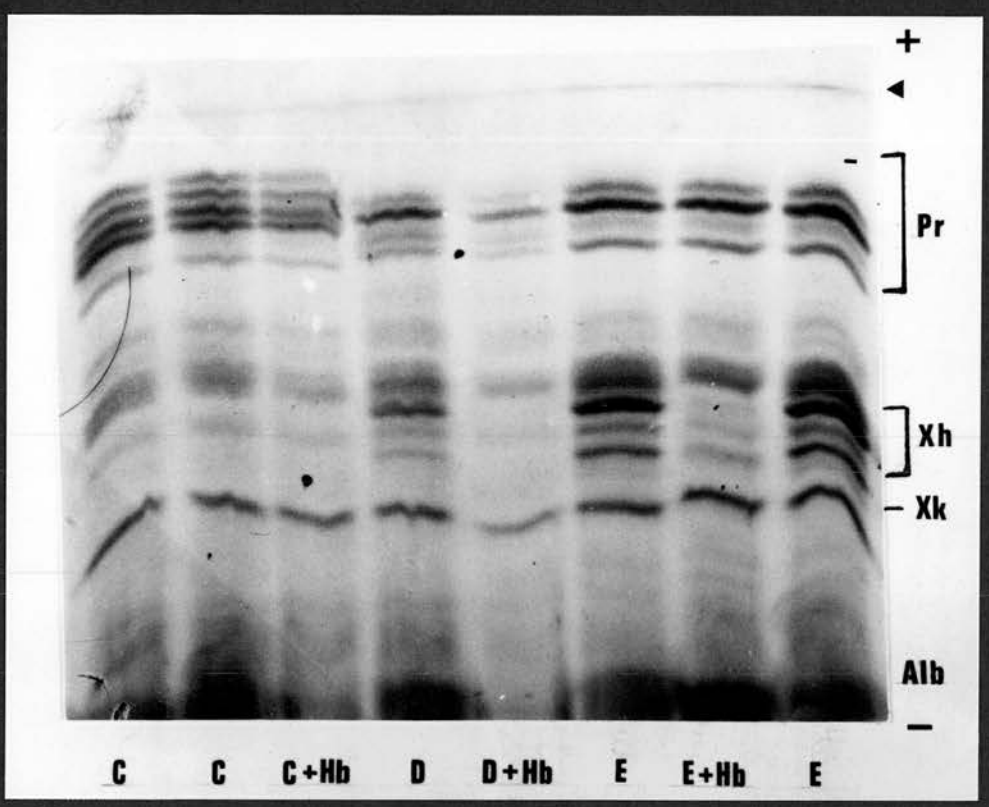
Alb - Indicates albumin.

Fig. 6.6. ASGE (pH 4.3) of horse sera C, D and E before and after incubation with haemoglobin (Hb).

◄ - Indicates borate boundary.

Alb - Indicates albumin.


+
Xh
Xk
Alb
–
A
A + Hb
B + Hb
B


+
Pr
Xh
Xk
Alb
–
C
C
C+Hb
D
D+Hb
E
E+Hb
E

particularly marked in the most anodal band. There is also a change in the conformation of the group of bands immediately anodal to the Xh zone.

(iv) Discussion

The results indicate that some of the bands within and immediately anodal to the so-called Xh system are able to bind haemoglobin to form a complex of slower Rf than the unbound protein. These bands correspond to haptoglobin.

Haptoglobin type 1-1 in human serum migrates in the prealbumin region after ASGE at pH 4.95 (Fagherol, 1972b). Since human and horse Hp/Hb complexes have a similar mobility after starch gel electrophoresis using Poulik's discontinuous buffer system (pH 8.6 - 8.9) (Braend and Effremov, 1965; Giblett, 1969) the appearance of haptoglobin in the acidic prealbumin region of horse serum is not surprising.

Although some of the bands in the Xh region appear to belong to a protein system other than haptoglobin, the number and identity of the proteins anodal to the so-called Xh system after ASGE remains to be resolved. To determine the number of antigenically distinct protein species in this region, antiserum was raised against the protein zones lying anodally to the so-called Xh region.

6.4.3. PREPARATION OF ANTISERUM AGAINST THE ANODAL PREALBUMIN PROTEINS AFTER ASGE (pH 4.3).

(i) Introduction

Antiserum against the prealbumin proteins anodal to the so-called Xh system after ASGE (pH 4.3) was prepared by a technique similar to that described by Martin et al., (1974) for the production of antiserum against human alpha-1 antitrypsin.

(ii) Materials and Methods

(a) Isolation of anodal prealbumins: Pooled horse serum was subjected to ASGE (pH 4.3) using a single insert extending across the width of the gel. The top slice of the gel and the lateral few centimetres of the bottom slice were discarded and the remaining gel was cut longditudinally into 2 segments. One segment was stained with 1% nigrosine. Using this marker, that portion of unstained gel anodal to the Xh system described by Braend (1967), including the borate boundary zone, was removed. After maceration in 5ml of sterile distilled water in a sterile Griffiths tube the gel was emulsified in 5ml of Freund's incomplete adjuvant. The remaining gel segment was stained with 1% nigrosine to check the exclusion of unwanted bands from the starch-adjuvant emulsion.

(b) Immunization of experimental rabbits: Adjuvant emulsions were prepared from 2 gels and each was inoculated subcutaneously into New Zealand White rabbits (R 92; R 95). A total of 10ml was inoculated over

4 sites on each rabbit. Prior to immunization both rabbits were bled and the serum separated and stored at -20°C. The rabbits were reimmunized on days 2 and 35 using fresh gel emulsions.

Both rabbits were bled on day 23 and their serum examined for specific antibody activity by agar gel immunoelectrophoresis (pH 8.6) against pooled horse serum using a continuous barbital-calcium lactate buffer system (pH 8.6) (Hirschfeld, 1960). 40ml of blood was harvested from each rabbit on days 25 and 51, and the separated serum was stored at -20°C.

(c) Isolation of immunoglobulin: The immunoglobulin fraction of the hyperimmune serum was isolated by saturated ammonium sulphate precipitation using the method of Stelos (1967). The precipitated immunoglobulins were concentrated by evaporation and subsequently dialysed for 12 hours against repeat changes of 0.01M PBS (pH 7.4). The concentrated immunoglobulins were stored at -20°C.

(d) Association of acidic prealbumins and albumin zone antiprotease activity: The relationship of anti-horse prealbumin immunoprecipitin arcs to the albumin zone of serum antiprotease activity was determined by simultaneous immunoelectrophoresis and trypsin digestion on fibrinagar gels (6.2.2).

(iii) Results

Immunoelectrophoresis of pooled horse serum

against serum harvested from R 92 and R 95 on day 23 resulted in 1 and 3 immunoprecipitin arcs respectively (Fig. 6.7.). Pre-immunization sera from both rabbits yielded no arcs. Serum from both rabbits harvested on day 51 resulted in 3 precipitin arcs against pooled horse serum.

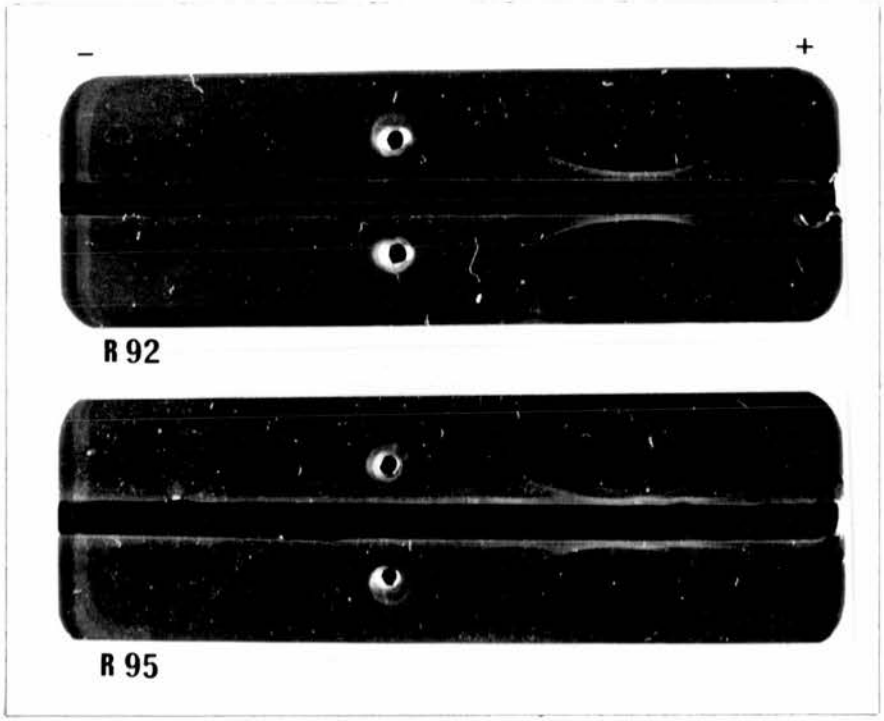


Fig. 6.7. Immunoelectrophoresis of pooled horse serum against rabbit anti-horse prealbumin serum harvested from R 92 and R 95 on day 23.

Immunoelectrophoresis of pooled horse serum against concentrated rabbit anti-horse prealbumin immunoglobulin prepared from serum harvested on days 25 and 51 showed 3 major precipitin arcs and at least 2 minor arcs (Fig. 6.8.). These arcs indicate the presence of at least 5 antigenically distinct

proteins in the region anodal to Xh after ASGE (pH 4.3).

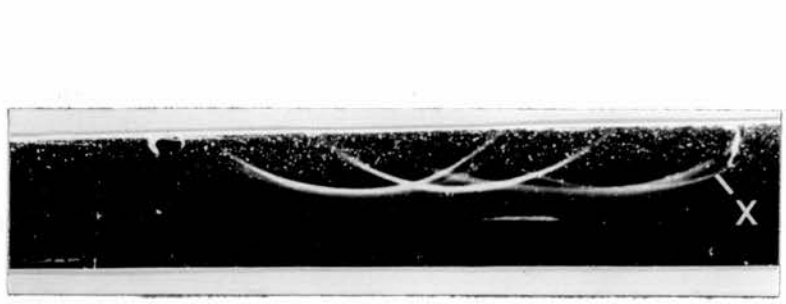


Fig. 6.8. Immunoelectrophoresis of pooled horse serum against concentrated rabbit anti-horse acidic prealbumin immunoglobulin.

Fibrinagar immunoelectrophoresis of pooled horse serum against concentrated anti-horse acidic prealbumin immunoglobulin in combination with trypsin digestion (6.2.2) showed that the most anodal of the three major precipitin arcs corresponded to the electrophoretically faster albumin zone antiprotease (Fig. 6.9). No arc corresponded to the electrophoretically slower antiprotease zone (Fig. 6.9).

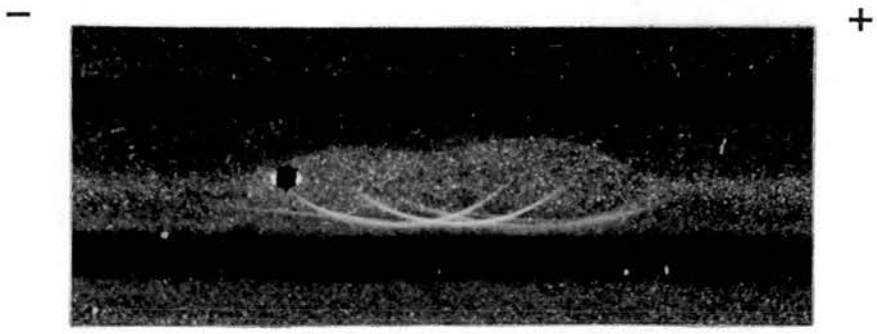


Fig. 6.9. Fibrinagar immunoelectrophoresis of pooled horse serum against concentrated rabbit anti-horse acidic prealbumin immunoglobulin (lower trough). The upper trough contains 0.032% ($^{w}/v$) bovine pancreatic trypsin in 0.0025N HCl.

(iv) Discussion

Two of the 3 major antigenically distinct protein species in the anodal prealbumin region after ASGE (pH 4.3) could correspond to the Pr protein and the aliesterase isozymes. These latter proteins, which are highly antigenic (Kaminski, 1978), lie immediately cathodal to the Pr system after ASGE (pH 4.8) (Scott, 1979). The third major protein probably corresponds to the haptoglobin bands appearing in the region anodal to the so-called Xh system (6.4.2). After immunofixation in conjunction with agarose electrophoresis (pH 8.6), the proteins corresponding to the 3 major anti-acidic prealbumin precipitin arcs can be shown to migrate in the albumin, alpha-1 and anodal alpha-2 regions (Section 6.6; page 179). These mobilities correspond to those of the Pr protein, aliesterases and haptoglobin respectively after zone electrophoresis (pH 8.6) (Gahne, 1966; Ek, 1977; this thesis, 3.2.2.).

The identity of the 2 minor protein species remaining has not yet been established. However the minor, most anodal arc (Fig. 6.8.X) may correspond to the protein observed at the borate boundary zone after ASGE (pH 4.3).

The most anodal of the major anti-acidic prealbumin precipitin arcs with an albumin zone mobility, corresponds to the electrophoretically faster anti-protease zone. Ek (1977) has shown that isolated

Pr protein migrates in the albumin zone after agarose electrophoresis (pH 8.6), suggesting that the Pr protein is the source of the albumin zone antiprotease activity in horse serum.

6.5. DIRECT DEMONSTRATION OF ANTITRYPSIN ACTIVITY ASSOCIATED WITH ANODAL PRE-ALBUMIN PROTEINS AFTER ASGE (pH 4.3).

6.5.1. INTRODUCTION

Inhibition of the proteolytic activity of trypsin by the prealbumin proteins after ASGE (pH 4.3) was demonstrated using a modification of the fibrinagar electrophoresis technique previously described (6.2.2). Inhibition of the esterolytic activity of trypsin by the prealbumin proteins was demonstrated by chromogenic ester substrate staining of the trypsin impregnated gel using the method described by Uriel and Berges (1968).

6.5.2. MATERIALS AND METHODS

(i) Inhibition of the Proteolytic Activity of Trypsin.

(a) ASGE (pH 4.3): ASGE of fresh horse sera was carried out as previously described (6.3.2).

(b) Inhibition of fibrinagar hydrolysis: Fibrinagar gels were prepared as previously described (6.2.2). Two parallel longditudinal troughs were cut

in the gels. A 0.032% ($^{w}/v$) solution of bovine pancreatic trypsin[1] in 0.0025N HCl was placed in one trough. The remaining trough was filled with a strip cut from the starch gel parallel to the direction of the run. The gels were then incubated overnight at 37°C. The remaining starch gel was stained with 1% nigrosine.

(ii) Inhibition of the esterolytic activity of trypsin.

(a) ASGE (pH 4.3): ASGE of fresh horse sera was carried out as previously described (6.3.2).

(b) Inhibition of chromogenic, ester substrate staining: A strip of starch gel was removed parallel to the direction of the run and immersed in 100ml of a 0.004% ($^{w}/v$) solution of bovine pancreatic trypsin[1] in 0.05M phosphate buffer (pH 7.4) and incubated at 37°C for 30 minutes. After washing in phosphate buffer, the gel was immersed in an ester substrate solution prepared as follows; 10 mg acetyl-DL-phenylalanine β napthyl ester[1] was dissolved in 4ml dimethyl formamide[1] and, immediately prior to use, was added to 36ml of a 0.05% ($^{w}/v$) solution of tetrazotized ortho-dianiside[1] in 0.05M phosphate buffer (pH 7.4). After incubation at 37°C for 30 minutes the gel was washed in 2% glacial acetic acid. Zones of inhibition of the esterolytic activity of the enzyme appeared as unstained areas against a diffuse pink background. The remaining starch gel

1. Sigma Chemical Co., Poole, England.

was stained with 1% nigrosine.

6.5.3. RESULTS

(i) Inhibition of the Proteolytic Activity of Trypsin.

Inhibition of trypsin hydrolysis of fibrinagar by prealbumin proteins after ASGE (pH 4.3) is shown in Figures 6.10.1. and 6.10.3. A discrete peak of inhibition of fibrinolysis (X) is associated with the most anodal prealbumin zones corresponding to the Pr protein. However, antiprotease activity extends cathodally from the Pr region towards the so-called Xh zone with a second, though less discrete, peak of activity (Y) corresponding to protein bands within the inter Pr-Xh region. A third discrete zone of antiprotease activity (Z) overlaps or lies cathodal to the Xk band.

(ii) Inhibition of the Esterolytic Activity of Trypsin

Chromogenic ester substrate staining of the acidic gels following incubation with trypsin is shown in Figures 6.10.2. and 6.10.3. A diffuse zone of negative staining appears at the anodal extremity of the gel in the region of the Pr system. The individual bands of the polymorphic Pr variants are not identifiable and the whole zone appears uniformly unstained. Areas of negative staining are not apparent in the inter Pr-Xh and Xh regions.

The chromatic staining method also demonstrates

Fig. 6.10.1. Antitrypsin activity of horse prealbumins after ASGE (pH 4.3)

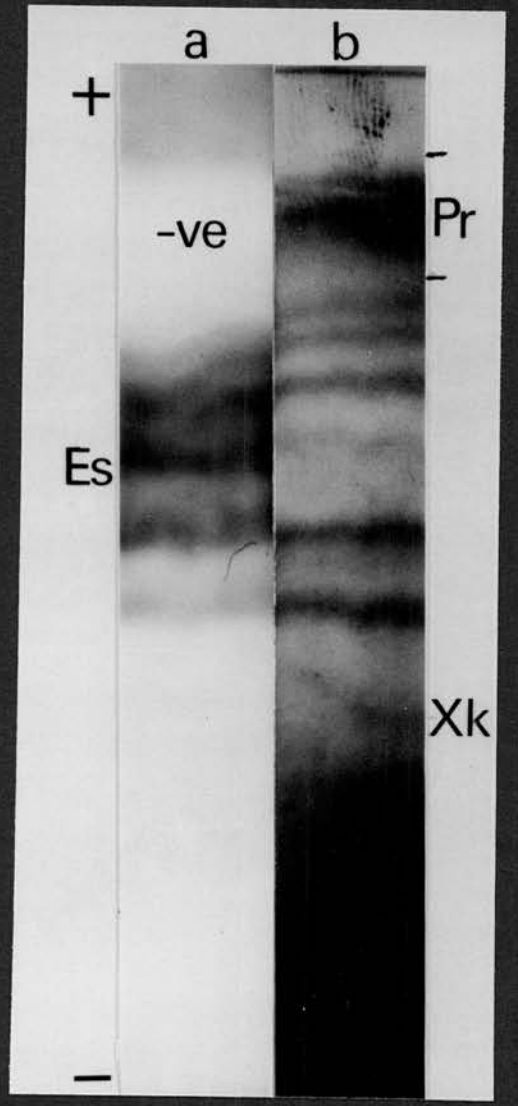
(a) Stained 1% nigrosine.

(b) Inhibition of fibrinolytic activity of trypsin - left hand trough contains bovine pancreatic trypsin.

Fig. 6.10. 2. Antitrypsin activity of horse prealbumins after ASGE (pH 4.3).

(a) Chromogenic ester substrate staining of trypsin impregnated gel. Inhibition of esterolysis is indicated by areas of negative staining (-ve).

(b) Stained 1% nigrosine.


a
b
+
-ve
Pr
Es
Xk
–

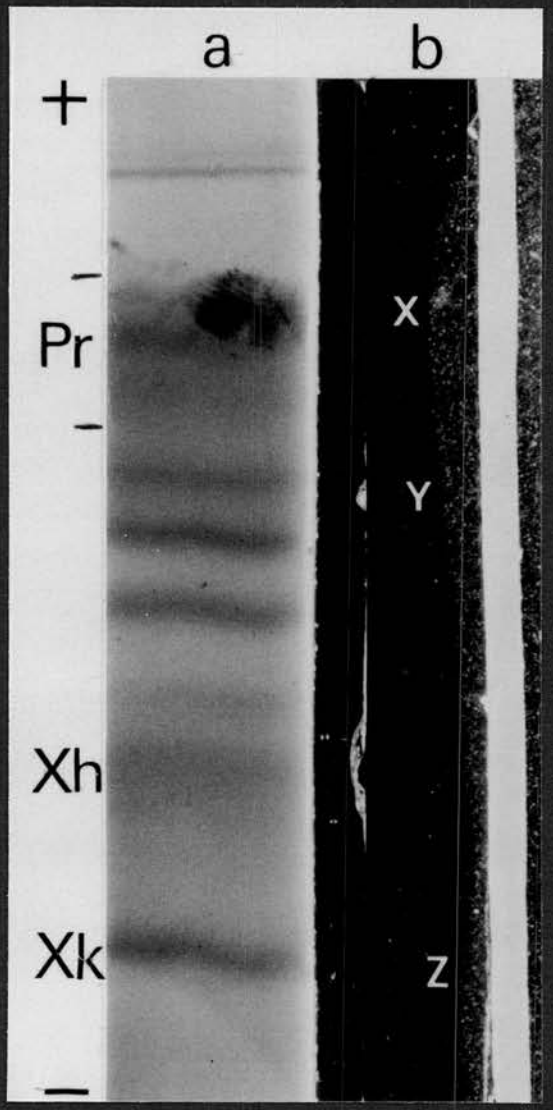
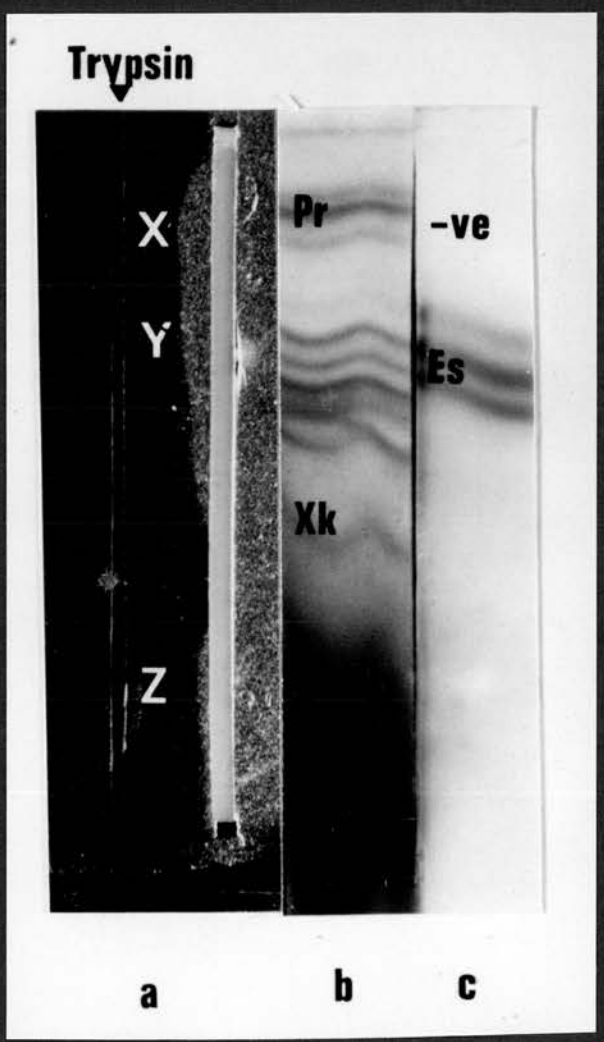

a
b
+
–
Pr
–
X
Y
Xh
Xk
Z
–

Fig. 6.10.3. Antitrypsin activity of horse pre-albumins after ASGE (pH 4.3).

(a) Inhibition of fibrinolytic activity of trypsin-left hand trough contains bovine pancreatic trypsin.

(b) Stained 1% nigrosine.

(c) Chromogenic ester substrate staining of the trypsin impregnated gel. Inhibition of esterolysis is indicated by areas of negative staining (-ve).


Trypsin
X
Y
Z
Pr
Xk
-ve
Es
a
b
c

the presence of the aliesterases isozymes (Es) in the inter Pr - Xh zone. In the case of electrophoretically slower esterase variants, the staining pattern extended into the Xh-Xk region. In one serum a single band of negative staining appeared in the albumin zone after ASGE, probably corresponding to the cathodal peak of antiprotease activity (Z) demonstrable using the fibrinagar technique.

When serum is subjected to both inhibition of fibrinagar digestion and chromogenic staining after ASGE the region of antiprotease activity extending cathodally from the Pr zone appears to correspond to the distribution of the aliesterase zones (Fig. 6.10.3).

6.5.4. DISCUSSION

Inhibition of trypsin mediated fibrinolysis in agar gels and the chromogenic ester substrate staining of trypsin impregnated starch gels have shown that inhibition of both the proteolytic and esterolytic activity of the enzyme is associated with the most anodal protein bands after ASGE which correspond to the highly polymorphic Pr locus (Braend, 1970). The Pr protein is thus a functional homologue of human alpha-1 antitrypsin.

The cathodal extension of antiprotease activity from the Pr system demonstrated by inhibition of fibrinagar hydrolysis (Figs. 6.10.1 and 6.10.3; Y) has also been demonstrated by Ek (1977) using a

similar, caseinagar based, technique. Ek (1979) subsequently showed that some of the protein bands in this cathodal region after ASGE (pH 4.8) were antigenically cross reactive with the Pr protein using an antigen-antibody crossed electrophoresis technique (Fig. 6.11). However, in his original study, Ek (1977) was unable to demonstrate binding of trypsin to proteins outwith the Pr system by incubation of serum with the enzyme prior to electrophoresis.

Inhibition of the esterolytic activity of trypsin appears restricted to the Pr system originally described by Braend (1970). Using Uriel and Berges's chromatic staining method, Pollitt and Bell (1980) recently confirmed the association of inhibition of the esterase activity of trypsin with the Pr system after polyacrylamide gel electrophoresis (pH 4.2 - 4.3) and, as in the present study, failed to demonstrate antiesterase activity cathodal to the Pr protein. However, in both studies the occurrence of substrate reactive aliesterases in this region may have masked the negatively stained zones which are indicative of antitrypsin activity.

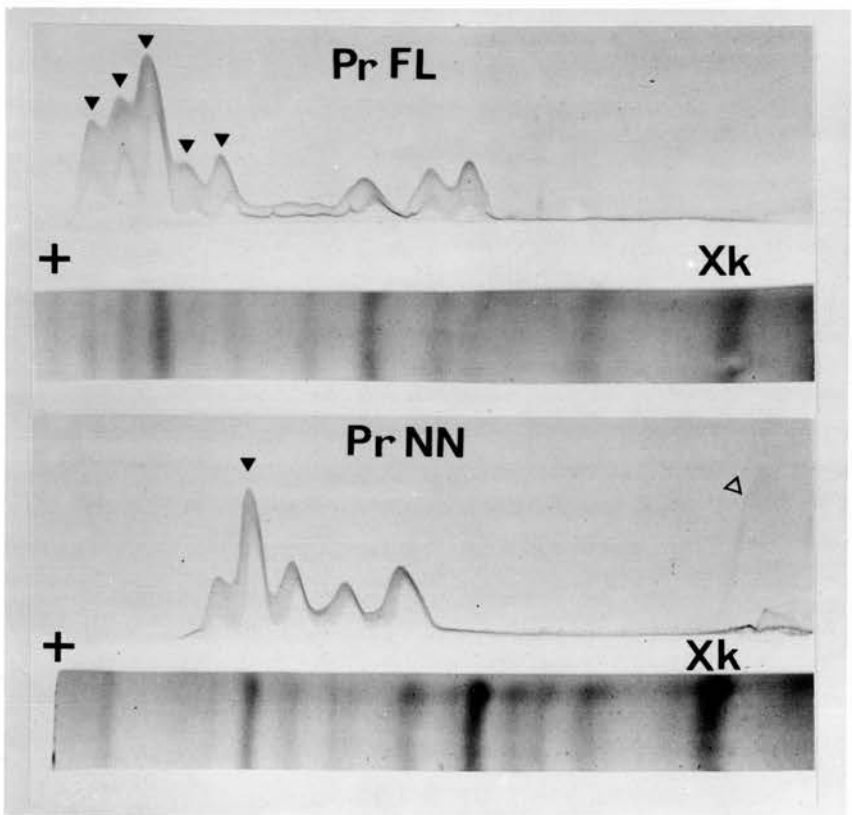
It is apparent that the cathodal extension of antiprotease activity from the Pr system after ASGE at pH 4.3 and pH 4.8 may be an independant antiprotease or may be due to previously unrecognised Pr allele products.

Fig. 6.11. Antigen-antibody crossed electrophoresis of horse prealbumin proteins after ASGE (pH 4.8) (Braend, 1970) developed against rabbit anti-horse Pr serum.

▼-indicates those bands diagnostic of the Pr phenotypes FL and NN after routine ASGE.

▽-indicates a Pr cross reactive protein in the Xk region.

The photographs are reproduced with the kind permission of Nils Ek.


Pr FL
+
Xk
Pr NN
+
Xk

In the former case the antiprotease could correspond to 1 of the 5 antigenically distinct proteins in this region. In Figure 6.10.3 the cathodal anti-protease peak Y corresponds to the aliesterase isozymes, suggesting that both may be associated.

Should these aliesterases possess protease inhibitory activity then the mechanism of inter-action with the protease appears similar in some respects to that of human alpha-2 macroglobulin. The macroglobulin strongly inhibits the proteolytic activity of the bound enzyme but has only limited inhibitory activity on its esterolytic activity. Furthermore the macroglobulin also possesses natural esterase activity which may arise from esterolytically active complexes formed with endogenous proteases (5.3.2.). The hypothetical association of the acidic aliesterases with antiprotease activity will be considered in greater detail in Part 4 of this chapter.

The minor, most cathodal zone of antiprotease activity (Figs. 6.10.1 and 6.10.3;Z) probably corresponds to the so-called Pr-aggregate described by Juneja, et al., (1979) and shown to occur in the Xk region after polyacrylamide gel electro-phoresis (pH 4.2 - 4.3) (Pollitt and Bell, 1980). Ek (1979; pers. comm.) has shown that this 'aggregate' is antigenically cross reactive with the Pr protein (Fig. 6.11).

PART 3

STUDIES OF THE MOLECULAR VARIANTS OF THE Pr ANTI-PROTEASE OF HORSE SERUM.

6.6. INTRODUCTION

Antiprotease activity has been shown to be associated with the most anodal group of serum protein bands after ASGE (pH 4.3). These bands appear to correspond to the allele products of the Pr locus (Braend, 1970). To examine this relationship the electrophoretic variants of the protein bands in the anodal prealbumin zone after ASGE (pH 4.3) were compared with the phenotypic Pr variants recognised after ASGE (pH 4.8) (Braend, 1970; Trommershausen-Smith and Suzuki, 1978a).

The Pr locus is defined by at least 9 autosomal codominant alleles (Braend, 1970; Scott, 1977), designated alphabetically according to their electrophoretic mobility; F, G, I, L, N, S, T, U and W. Scott (1976) has calculated that the Pr protein is the single most effective electrophoretic polymorphism for detecting falsely assigned parentage in horses. Nevertheless, the technical difficulties involved in Pr phenotyping by ASGE (Scott, 1977) have limited its use in this field. Of the 22 participant laboratories in the 1977 Horse Blood Typing Comparison Test only 7 phenotyped the Pr system, and amongst these 7 laboratories there was very limited agreement on the Pr phenotypes of the test sera (Storset and Braend, 1979; pers. comm.).

For these reasons the genetic polymorphism of the Pr protein was also examined using isoelectric focusing and immunofixation after agarose electrophoresis (pH 8.6). Both these techniques have been used for phenotyping alpha-1 antitrypsin variants in human serum (5.3.1, vi).

6.6.1. ACIDIC STARCH GEL ELECTROPHORESIS (ASGE)

(i) Introduction

ASGE (pH 4.3) was used to examine the Pr polymorphism within a family based population of Thoroughbred horses and in a random population of mixed bred horses. The study was designed to compare the phenotypic appearance of the Pr variants after ASGE (pH 4.3) with those reported after ASGE (pH 4.8).

(ii) Materials and Methods

(a) Sera: Sera from 37 sire - dam - offspring groups made up of 70 English Thoroughbred (TB) horses were obtained from 4 studs. A further 30 sera were obtained from horses and ponies of mixed breeding. All sera were stored at -20°C for up to 2 years until used.

(b) Electrophoresis: ASGE (pH 4.3) (6.2.1.(ii)) was carried out twice on all sera and the gels were stained using 1% nigrosine. As far as possible family groups were simultaneously electrophoresed.

To check Pr phenotype diagnosis after ASGE (pH 4.3) the Pr phenotypes of the 30 sera from horses of mixed breeding were also determined using ASGE (pH 4.8) by

Dr. Mikael Braend in the Blood Typing Laboratory of the Veterinary School of Norway using the method of Braend (1970).

(c) Statistical Methods: The null hypothesis of absence of differences in Pr allele frequencies between different populations was tested using the G-test (Sokal and Rohlf, 1973; Buis, 1976). This test is described in detail in section 8.2.1.

(iii) Results

Examples of the acidic gels (pH 4.3) used in this study are shown in Figures 6.13 to 6.16.

Codominantly inherited polymorphism of the most anodal acidic prealbumin protein bands of TB serum was apparent after ASGE (pH 4.3). Within the TB population, presumed homozygous allele products were observed which were identical to the PrL and PrU homozygotes after ASGE (pH 4.8) (Braend, 1970; Trommershausen-Smith and Suzuki, 1978a). Typical PrF, I, L and U products after ASGE (pH 4.8) were recognised in the heterozygous form in individuals within the TB population. However, the relative mobilities of the PrN and PrS products after ASGE (pH 4.3) differed from those reported after ASGE (pH 4.8). Both these products adopted a more anodal mobility at pH 4.3 than at pH 4.8 and the PrN product almost overlapped the major PrL band. The band pattern and relative mobilities of the homozygous and heterozygous Pr

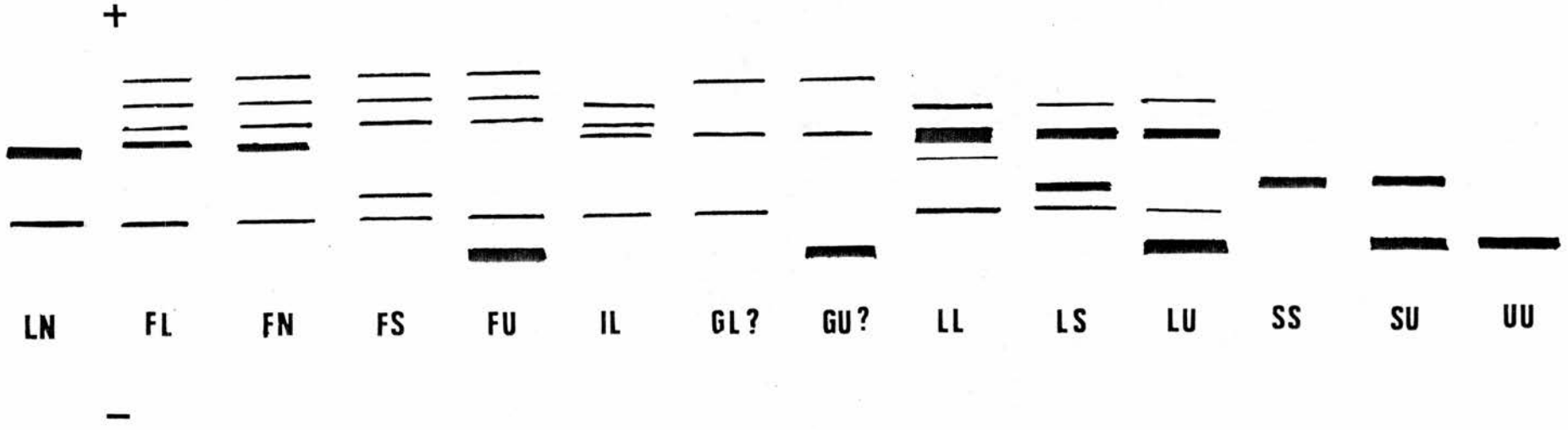
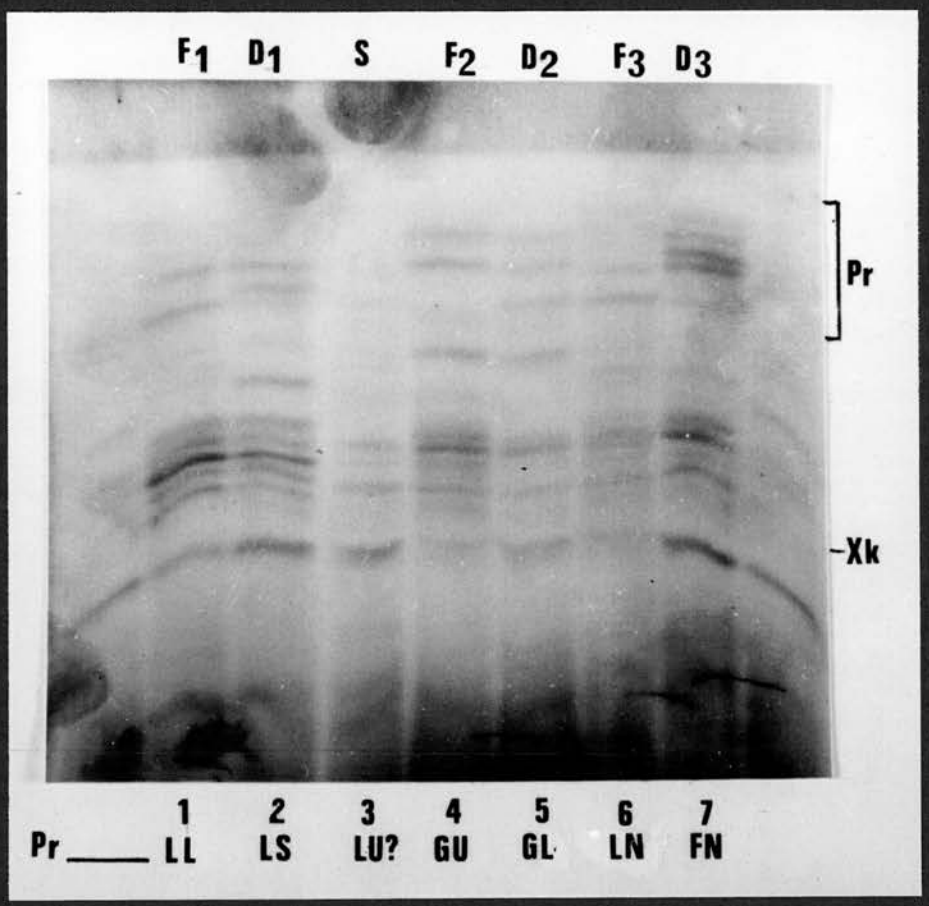


Fig. 6.12 Schematic representation of the Pr phenotypes observed in the Thoroughbred population after ASGE (pH 4.3).

Fig. 6.13. ASGE (pH 4.3) of Thoroughbred sera.

Fig. 6.14. ASGE (pH 4.3) of Thoroughbred sera.


F1 D1 S F2 D2 F3 D3
Pr
Xk
1 2 3 4 5 6 7
Pr LL LS LU? GU GL LN FN

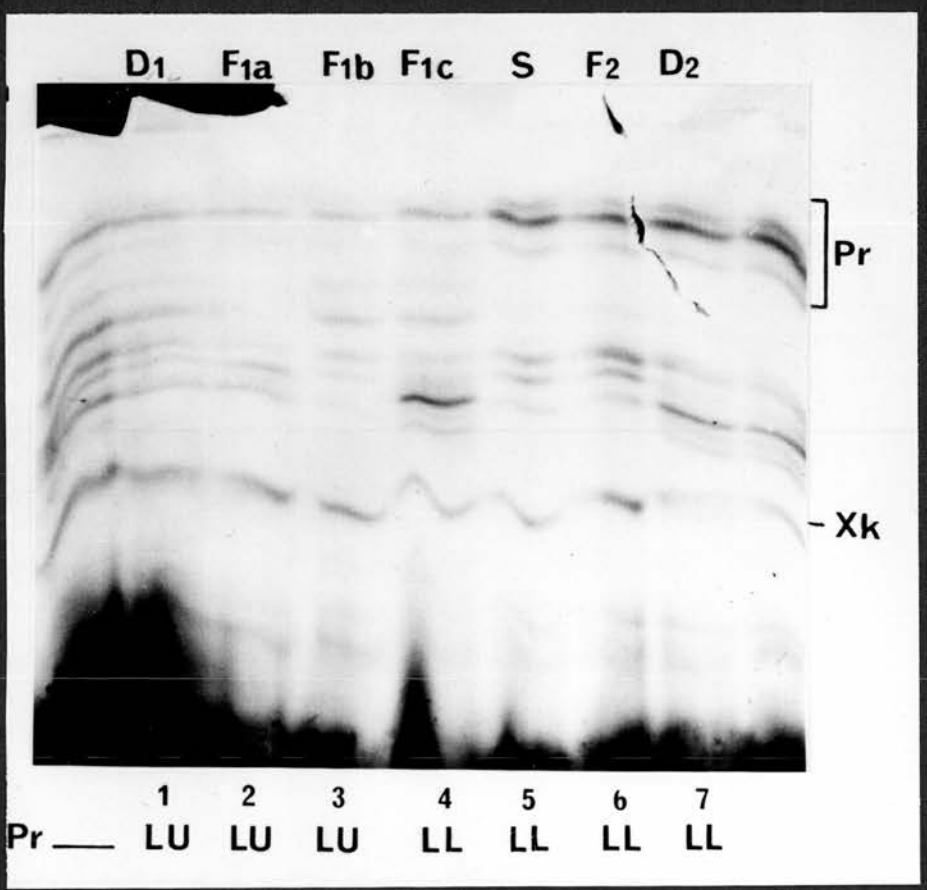
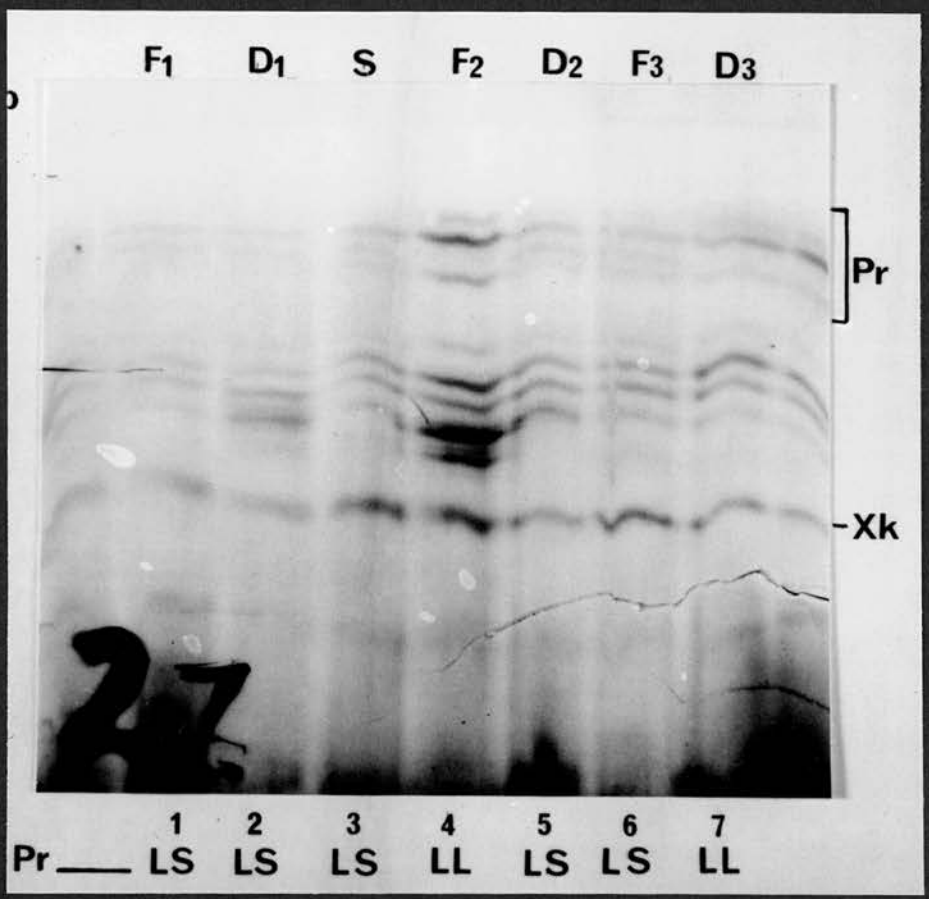
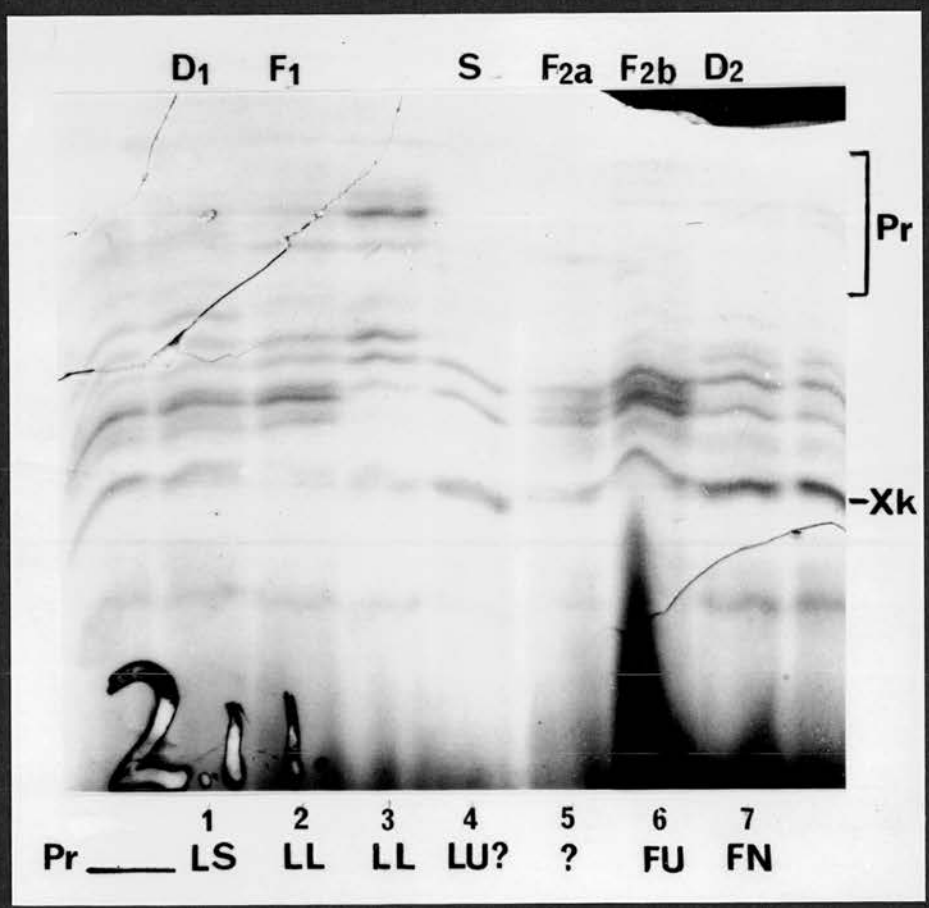

D1 F1a F1b F1c S F2 D2
Pr
Xk
1 2 3 4 5 6 7
Pr LU LU LU LL LL LL LL

Fig. 6.15. ASGE (pH 4.3) of Thoroughbred sera.

Fig. 6.16. ASGE (pH 4.3) of Thoroughbred sera.


F1 D1 S F2 D2 F3 D3
Pr
Xk
1 2 3 4 5 6 7
Pr LS LS LS LL LS LS LL


D1 F1 S F2a F2b D2
Pr
Xk
1 2 3 4 5 6 7
Pr LS LL LL LU? ? FU FN

phenotypes within the TB population after ASGE (pH 4.3) are shown schematically in Figure 6.12. Included in Figure 6.12 are the presumed PrG allele products (Scott, 1976) which were observed in a single dam-offspring group (Fig. 6.13).

The appearance of Pr phenotypes within the TB population after ASGE (pH 4.3) are shown in Figures 6.13 to 6.16. The Pr type and family status of the individual sera are presented. The notation used in these Figures is as follows; S-Sire, D-Dam, F-Foal; 1, 2... denotes dams and foals within a single mating group; a, b, c... denotes foals derived from the same sire-dam group.

Comparison of the Pr phenotypes of the 30 sera from horses of mixed breeding determined by ASGE (pH 4.3) with those determined by Dr. Braend using ASGE (pH 4.8) are shown in Table 6.2. The PrW product was not observed in the TB population. However, within the mixed population the PrW product after ASGE (pH 4.3) was identical to that after ASGE (pH 4.8). Complete agreement in Pr phenotype was observed in 20 of the 30 sera. Of the 10 misdiagnosed phenotypes, 8 involved failure to recognise the PrN and/or the PrI product. However, these products were correctly identified in other sera. This comparison test confirms the identity of the Pr allele products observed after ASGE (pH 4.3).

There was considerable variation in both the degree of separation and the staining intensity of the

TABLE 6.2.

COMPARISON OF Pr PHENOTYPES IN 30 SERA DETERMINED BY ASGE (pH 4.3) AND BY ASGE (pH 4.8) (BRAEND, 1970).

HORSE	ASGE (pH 4.3)	ASGE (pH 4.8) (Braend, 1970)	Discrepancy
1	IS	IS	
2	NW	LW(atypical W product)	*
3	LS	IL	*
4	LL	LN(No N product when checked)	
5	FL	FL	
6	LL	LL	
7	LL	LL possibly LS	
8	LW	LW	
9	FS	FS	
10	LL	LL	
11	LL	LL	
12	IS	IS	
13	LS	LS	
14	LS	FS	*
15	FL	FL	
16	LS	NS	*
17	LL	NS	*
18	NS	NS	
19	IU	IS	*
20	FL	FL	
21	SW	SU(atypical U product)	
22	SS	IS (weak I product)	*
23	LL	LL	
24	LL	IN	*
25	LL	LN	*
26	SU	IS	*
27	IL	IL	
28	LL	?	
29	LS	LS	
30	FL	FL	

Pr bands between gels. This was also observed by Braend (1970). In some sera the expression of the allele products was so weak as to preclude phenotype diagnosis. This is illustrated in Figures 6.13S and 6.16S where the presumptive Pr phenotypes of this sire is LU. This is based upon 4 mare-offspring groups although the allele expression is insufficient to confirm this.

The Pr phenotypes diagnosed within the TB population are presented in Table. 6.5a (p.164). Only those types which were substantiated by complete family data are given. The pattern of inheritance amongst the 12 mating classes within the TB population, comprising of 6 stallions and 22 mares, are shown in Table 6.3.

TABLE 6.3.

INHERITANCE OF Pr PHENOTYPES OF THOROUGHBRED HORSES IN DIFFERENT MATING CLASSES.

MATING CLASSES		NUMBER OF OFFSPRING/PHENOTYPES
1	LL x LL	3/LL
2	LL x IL	2/IL 1/LL
3	LL x LU	1/LL 4/LU
4	LU x LS	1/LL 1/SU
5	LU x SU	2/UU 1/SU
6	LU x FL	1/LU 2/FU
7	LS x FN	1/LN
8	LS x FS	1/FS
9	LS x LS	2/LL 1/LS
10	LS x LL	2/LS 3/LL
11	LS x SU	1/SS
12	LU x IL	1/IL 1/LL

The Pr allele frequencies within the TB population are shown in Table 6.4. Allele frequency is expressed as $\frac{n}{2N}$; where n = number of allele occurrences and N = number of individuals in the population. The Pr allele frequencies within TB populations reported by Scott (1976) and Blokhuis and Buis (1979) using the ASGE (pH 4.8) method of Braend (1970) are also indicated in Table 6.4.

TABLE 6.4.

FREQUENCY OF Pr ALLELES OCCURRING IN THOROUGHBRED POPULATIONS.

Allele \ Author	Present Study N = 62	Scott (1976) N = 1500	Blockhuis & Buis (1979) N = 40
F	0.081	0.057	0.05
G	-	0.030	-
I	0.040	0.077	0.07
L	0.532	0.447	0.54
N	0.024	0.177	0.05
S	0.161	0.079	0.29
T	-	-	-
U	0.161	0.133	-
W	-	-	-

Blokhuis and Buis (1979) did not recognise a PrU allele in their series and so their results are not comparable with those of the present study or of Scott (1976). However significant differences occurred

between the Pr allele frequencies within the TB populations of Scott and those of the present study ($G = 46.37$, $d.f. = 6$; $P < 0.005$), particularly PrN and PrS allele frequencies.

(iv) Discussion

The Pr antiprotease allele products after ASGE (pH 4.3) are similar to those after ASGE (pH 4.8) (Braend, 1970; Trommershausen-Smith and Suzuki, 1978a). The exception is the PrN and PrS products where pH dependant variation in relative electrophoretic mobilities were observed. The relatively faster mobilities of the PrN and PrS products at the more acidic pH have been observed by Pollitt and Bell (1980) using polyacrylamide gel electrophoresis (PAGE). These authors showed that after PAGE (pH 4.2 - 4.3) the relative mobilities of the PrN and PrS products were similar to those after ASGE (pH 4.3), the major PrN product being identical to the major PrL product. The relative mobilities of the remaining Pr allele products after PAGE (pH 4.2 - 4.3) were the same as after ASGE (pH 4.8). The relatively faster mobility of the PrN and S products at pH 4.3 suggests that their molecular structures may be susceptible to dissociation below pH 4.8.

Comparison of the Pr phenotypes of 30 sera of horses of mixed breeding diagnosed using ASGE (pH 4.3) with those diagnosed by Braend highlight the difficulties in recognising the PrN and PrI products after ASGE

(pH 4.3). Failure to recognise the PrN product probably arose from confusion with the major PrL product, the two being almost indistinguishable after ASGE pH 4.3. The cause of the failure to recognise the PrI product is not known, although in a number of cases it may have resulted from weak allele expression.

The extent of differences in Pr phenotype diagnosis between the present method and that of Braend is 33%. However this figure compares favourably with the errors of 3%, 20%, 38%, 57% and 69% amongst 5 laboratories reporting the Pr system during the 1979 Horse Blood Typing Comparison Test (Juneja, 1980; pers. comm.). These figures probably reflect the technical difficulties involved in using this system.

Comparison of the allele frequencies within the T.B. population in the present study with those of Scott (1976) show a reversal of PrN and PrS frequency. This may have resulted from the poor separation of the PrN and PrL products at pH 4.3. However it may also be due to the relatively low numbers of non-randomly selected TB horses used in the present study, resulting in a biased distribution of alleles.

Scott (1979) proposed that the Pr system be extended cathodally to include bands within the inter Pr-Xk zone. This suggestion is supported by the antigen-antibody crossed electrophoretic data of Ek (1979) (Fig. 6.11). However Ek (1979) showed that after ASGE (pH 5.4) the Pr protein migrated as a

single band and that both the inherited polymorphism of the Pr protein and the more cathodal Pr cross reactive bands only became apparent after ASGE at more acidic pH. Furthermore Ek (1977) had earlier shown that at pH 4.8 only those Pr bands originally described by Braend (1970) were removed after incubation with trypsin at concentrations less than the serum trypsin inhibitory capacity (5.4.2.). These observations suggest that the 'cathodal' Pr cross reactive bands may be non-functional acid dependant aggregates of the Pr proteins. Such aggregate or polymeric forms of human alpha-1 antitrypsin have been described, although prolonged incubation at low pH is necesssry for their formation (Glaser, Karic and Cohen, 1977).

The difficulties encountered in Pr phenotyping using the ASGE technique in the present study and also by other workers routinely carrying out Pr phenotyping prompted a study of the use of isoelectric focusing as an alternative means of phenotyping the antiprotease.

6.6.2. ISOELECTRIC FOCUSING OF HORSE ACIDIC PREALBUMINS ON THIN LAYER POLYACRYLAMIDE GELS.

(i) Introduction

Isoelectric focusing has been defined by Righetti and Drysdale (1976) as an equilibrium electrophoretic method for segregating amphoteric macromolecules according to their isoelectric points in stable pH gradients. Although the principles of isoelectric separation have been known for some time, difficulty in maintaining a stable pH gradient held up development.

Then Svensson (1962) and Vesterberg and Svensson (1966) described 'natural' pH gradients formed by passing an electric current through a solution of low molecular weight amphoteric molecules (ampholytes) such as protein hydrolysates and synthetic peptides. Ideally ampholytes should possess appreciable conductance in their isoelectric state, a property always accompanied by good buffering capacity (Vesterberg and Svensson, 1966). Synthetic ampholytes possessing these properties were produced by Vesterberg (1969) and have now become commercially available. These result in smooth and stable gradients over a variety of pH ranges. The principles of electrofocusing and the properties of carrier ampholytes have been discussed in detail by Righetti and Drysdale (1976). Vesterberg (1973a) described a method of analytical electrofocusing on thin layer polyacrylamide gels. More recently, techniques of analytical electrofocusing on flat beds of granulated gels such as agarose acrylamide have been described (Johanssen and Hjerten, 1974).

To overcome the difficulties associated with the ASGE technique, routine phenotyping of human alpha-1 anti trypsin variants is carried out in many laboratories using polyacrylamide gel isoelectric focusing (PGIEF) within the pH range 3.5 - 6.0. PGIEF provides a reproducible method for the direct diagnosis of alpha-1 antitrypsin phenotypes without resorting to crossed electrophoresis. The appearance of the alpha-1 antitrypsin variants on ASGE and PGIEF are similar,

although greater resolution of the allele products is achieved with the latter technique (Arnaud et al., 1974).

Fisher and Scott (1978) have reported similar phenotypic aliesterase isozyme patterns of horse serum after PGIEF (pH 3.5 - 5.5) and ASGE (pH 4.05). Furthermore, neuraminidase treatment of horse serum has shown that a factor determining electrophoretic mobility of both the aliesterase isozymes and the Pr variants is net molecular charge (Gahne, 1966). Thus the phenotypic appearance of the Pr proteins after ASGE should be reproduced after PGIEF.

With the exception of that of Fisher and Scott (1978), there have been no reports of PGIEF being used to study horse serum protein polymorphisms.

In this section a PGIEF technique allowing separation of the acidic prealbumin proteins of horse serum comparable to that after ASGE is described. The distribution of the major prealbumins, Pr haptoglobin and the aliesterases after PGIEF is then determined, followed by a study of the Pr phenotypes after PGIEF. Lastly application of the technique to Pr phenotyping is discussed.

(ii) Materials and Methods

(a) Sera: The T.B. sera whose Pr types have been previously determined by ASGE (pH 4.3) (6.6.1)

were used. In addition, the 30 sera of horses of mixed breeding whose Pr phenotypes were determined by Dr. Braend of the Veterinary School of Norway (Table 6.2) were also used.

(b) Electrofocusing: Thin-layer polyacrylamide gel electrofocusing (pH 4.0 - 6.0) was carried out using the LKB 2117 Multiphor system[1]. The gel frames (250 mm x 125 mm) were made up of a thick glass plate (LKB 2117 - 105[1]), a thin glass plate (LKB 2117 - 104[1]), a rubber gasket (LKB 93 90 6010[1]) and a second thick glass plate held together with bulldog clips. The gel contact surfaces were thoroughly cleaned with detergent, dried and wiped with ethanol before use.

Three gel stock solutions were used:

(1) Acrylamide: 58.1 g acrylamide dissolved in 200ml distilled water, passed through a 0.22 µm-pore size filter[2] and stored at 4°C;

(2) NN' - methylenebisacrylamide: 1.8 g bisacrylamide dissolved in 200 ml distilled water, passed through a 0.22 µm-pore[2] size filter and stored at 4°C.

(3) Riboflavine: a saturated solution in distilled water was prepared and stored at 4°C.

The gel was prepared by mixing 13 ml acrylamide and 13 ml bisacrylamide with 30 ml of 25% (w/v) sucrose solution, to which 3.5 ml of sterile ampholine, pH 4.0 - 6.0 (LKB 1809 - 116) was added. To this mixture 0.4 ml

1 LKB Produckter, Bromma, Sweden.

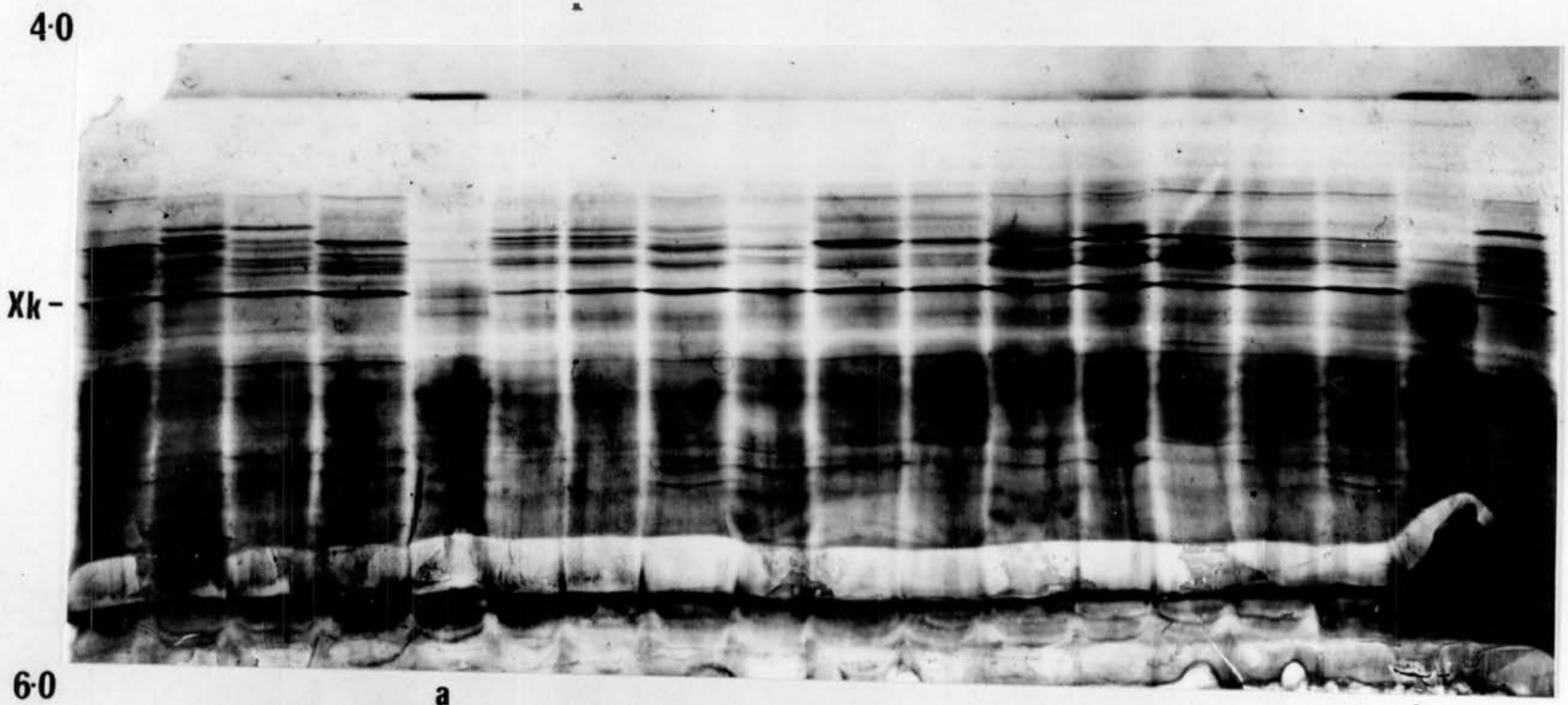
2 Millipore Corporation, Molshiem, France.

riboflavine solution was then added as a polymerising agent. The mixture was degassed by strong suction into a 50 ml plastic syringe through a 19-gauge needle and the mixture then poured into the frame. Care was taken to exclude air from the frame during pouring. The gel was allowed to polymerise at room temperature under ultraviolet light. Polymerisation was usually completed within 2-3 hours, indicated by a change in refractive index at the periphery of the gel. The gels were stored overnight at 4°C.

After removal of the two thick glass plates approximately 5 µl of serum on rectangular strips of LKB inserts (2117-106), were applied along the length of the gel, 1 cm from the cathodal wick, using a paper template as a guide. The anodal and cathodal wicks were applied to the gel after soaking in 1M phosphoric acid and 1M sodium hydroxide respectively, giving an electrode distance of 100 mm.

The gel on the thin glass plate was placed upon a layer of water laid over the cooling plate of the Multiphor. The focusing lid which allowed voltage application across the breadth of the tank was then fitted, ensuring good contact between the wicks and the gel surface. After starting the cooling system the power pack was set to deliver a maximum of 1000 V and 30 W. The initial potential difference of 400V increased to maximum during the run and the initial current of 30 mA fell as the pH gradient formed.

Fig. 6.17. PGIEF (pH 4.0-6.0) of horse sera using a 2 hour focusing time, showing the prealbumin protein distribution in relation to the Xk band.
(a) and (b) are human sera of alpha-1 antitrypsin (Pi) phenotype MZ.


4·0
Xk -
6·0
a
b

Electrofocusing was completed after 6-hours.

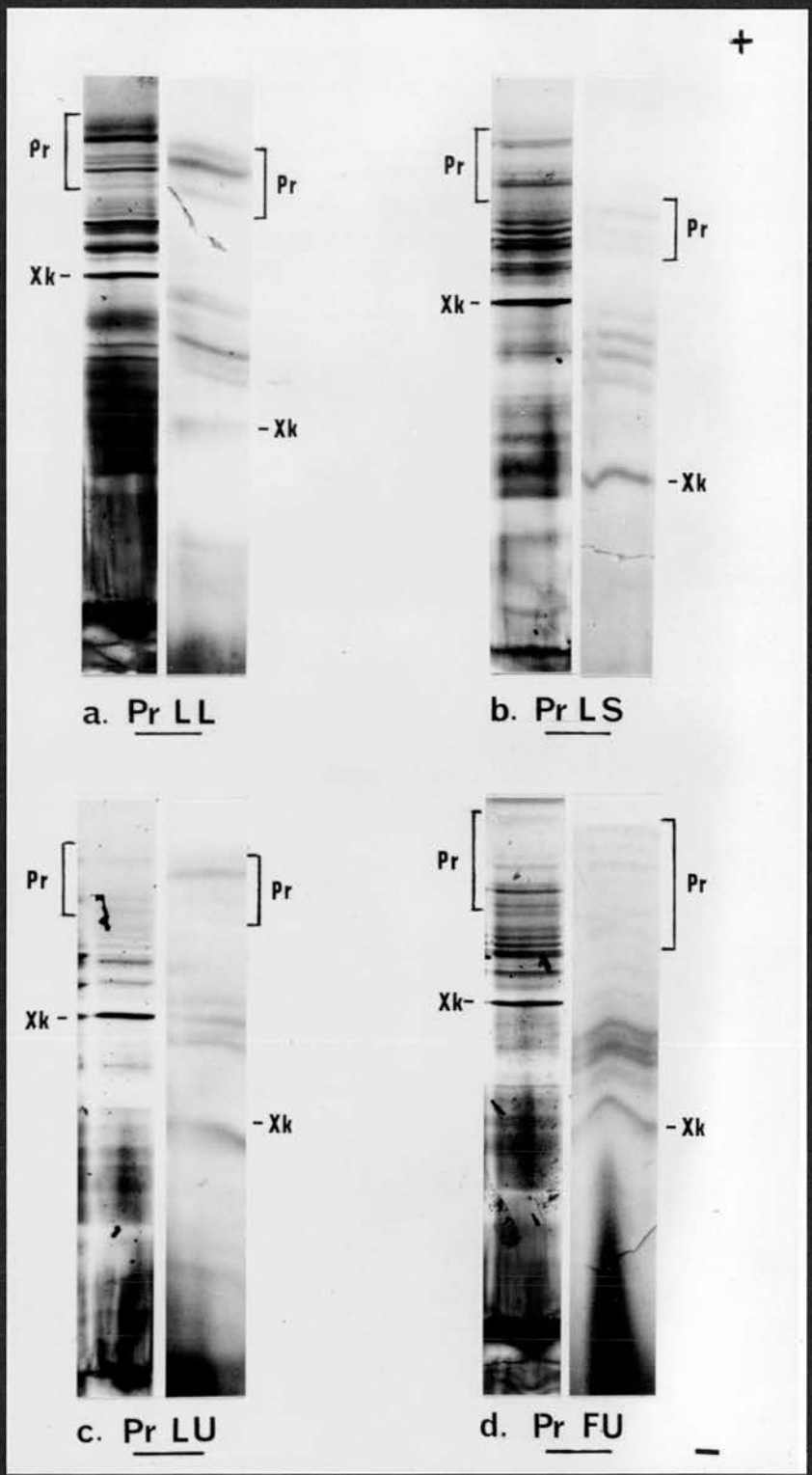
The gels were stained for 30 minutes at 60°C with Coumassie Brilliant Blue (Vesterberg, 1973a). Destaining took place overnight using a 8:3:1 water/ethanol/acetic acid solution. The gels were mounted for prolonged storage in a glycerine/destaining solution (1:4) mixture.

(iii) Results

The 2 hour focusing time recommended by Vesterberg (1973a) for PGIEF under the conditions described above resulted in poor resolution of the more anodal prealbumin protein bands of horse serum. This is shown by comparing the prealbumin zones after a 2 hour run (Fig. 6.17) and after a 6 hour run (Figs. 6.24; 6.25). The longer time was necessary to achieve complete resolution of the more anodal proteins. In contrast, the increased focusing time did not markedly affect the resolution of the major alpha-1 antitrypsin bands of human serum, as shown in a Pi MZ phenotype in Figure 6.17, a and b and Figure 6.24, 20.

The appearance of the prealbumin protein bands after PGIEF (pH 4.0 - 6.0) is similar to that after ASGE (pH 4.3) (Fig. 6.18). However, the increased resolution achieved using PGIEF resulted in focal concentration of protein bands. Thus, single diffuse bands after ASGE appeared as discrete multiple bands after PGIEF. Furthermore, the results

Fig. 6.18. Comparison of the appearance of the prealbumin proteins of horse serum after PGIEF (pH 4.0 - 6.0) and ASGE (pH 4.3). Pr phenotypes LL, LS, LU and FU are shown.


+
Pr
Xk
Pr
Xk
a. Pr LL
Pr
Xk
Pr
Xk
b. Pr LS
Pr
Xk
Pr
Xk
c. Pr LU
Pr
Xk
Pr
Xk
d. Pr FU

achieved using PGIEF were reproducible and band distortion was rarely encountered.

Comparison of horse and human serum after PGIEF (pH 4.0 - 6.0) (Fig. 6.24) illustrates the relative complexity of the prealbumin zones of horse serum. The multiplicity of equine prealbumin bands after PGIEF (pH 4.0 - 6.0) neccessitates identification of individual proteins according to their isoelectric mobility. Accordingly, the distribution of the major acidic prealbumin proteins was determined after PGIEF (pH 4.0 - 6.0).

(iv) Distribution of prealbumin proteins after PGIEF (pH 4.0 - 6.0) of horse serum.

(a) Introduction: By analogy with ASGE (pH 4.3) at least 5 antigenically distinct acidic prealbumin proteins may be expected to appear in the region anodal to the distinct Xk protein after PGIEF (pH 4.0 - 6.0). These include haptoglobin, the aliesterases and the Pr antiprotease. A single protein band appearing at the acidic extremity of the gel after PGIEF probably corresponds to the borate boundary protein after ASGE (pH 4.3).

(b) Materials and Methods: The distributions of the 3 major acidic prealbumin proteins after PGIEF were determined as follows:-

Haptoglobin: Purified horse haptoglobin, isolated

by gel filtration chromatography and Pevikon block electrophoresis, was kindly donated by Peter Johnstone of the Moredun Institute, Edinburgh. This was subjected to PGIEF (pH 4.0 - 6.0) and then stained with Coumassie Blue.

Aliesterases: Esterase activity was demonstrated by immersing the gels in a solution containing 12ml of 1% ($^{w}/v$) alpha napthyl acetate[1] in 50% acetone and 200mg of Fast Blue^{B1} in 200mls 0.05M phosphate buffer (pH 6.8). Once sufficient colour had been generated the gels were transferred to a 1:5:5 solution of glacial acetic acid / methanol /water.

Pr antiprotease: Since the substrate reactive aliesterases may interfere with the chromatic ester substrate staining method of identifying antiprotease activity after electrophoresis, the antiprotease activity of the prealbumin proteins after PGIEF was determined using the 'protease probe' method. Allen, et al., (1977) used this method to demonstrate antiprotease activity associated with alpha-1 antitrypsin variants after PGIEF of human serum. The method depends upon the formation of inhibitor-enzyme complexes of higher pI than the free inhibitor. Human alpha-1 antitrypsin readily combines with proteases to form complexes of pI around 6.0 (Pannell, Johnson and Travis, 1974; Saklatvala et al., 1976).

1 Sigma Chemical Co., Poole, England.

Doubling dilutions of bovine pancreatic trypsin[1] in 0.01M PBS (pH 7.4) were added in a 1:9 ratio to 1ml aliquots of 3 horse sera of Pr phenotypes LL, LU and SW, resulting in a final concentration of 12.5mg, 6.25mg, 3.12mg, 1.56mg and 0.78mg enzyme per ml serum. The mixtures were incubated at 37°C for 30 minutes and the pH of each determined using a pH-blood gas analyser[2]. The mixtures, excluding the 12.5mg/ml enzyme-serum dilutions, along with serum-PBS controls were subjected to PGIEF (pH 4.0 - 6.0).

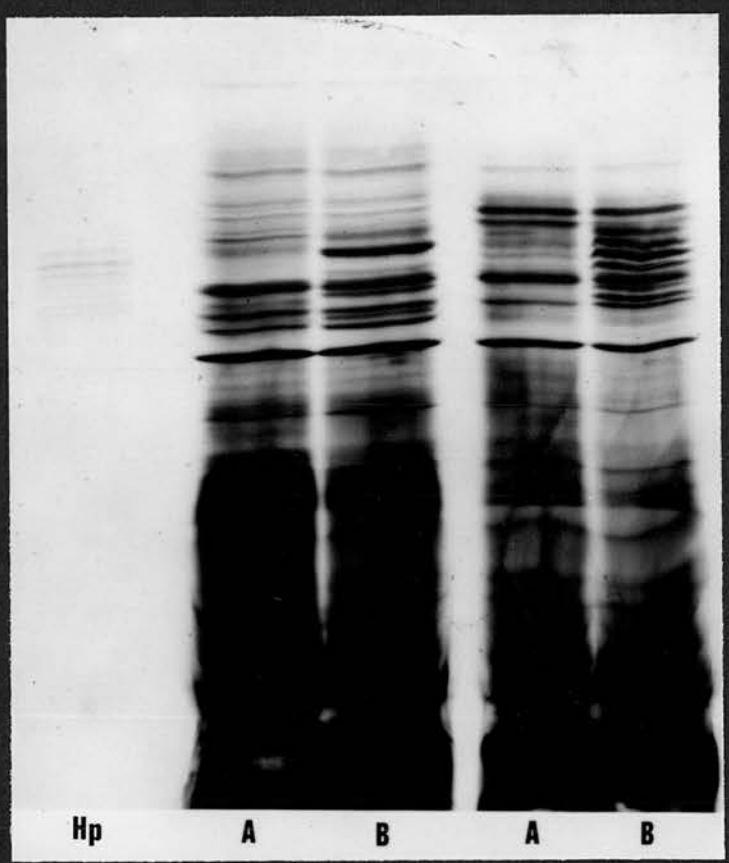
(c) Results: Since the pH gradient across thin layer gels is effectively linear (Allen et al., 1977) the pIs of the individual prealbumin proteins may be estimated by direct measurement.

Haptoglobin: Horse haptoglobin is a highly polymorphic protein of pI 4.4 - 4.55. Isolated haptoglobin after PGIEF (pH 4.0 - 6.0) is shown in Figure 6.19 along with whole sera of two horses taken before (A) and after (B) the onset of paralytic ileus. These sera show marked changes in prealbumin banding patterns associated with this disease. These prealbumin changes are partly due to haptoglobin changes (Johnstone, 1980; pers. comm.).

Aliesterases (Es): These are highly polymorphic isozymes whose molecular variants appear to be determined by at least 7 autosomal codominant alleles (Fisher and Scott, 1978). The appearance of aliesterase isozymes

1. Sigma Chemical Co., Poole, England.
2. Corning Instruments, U.S.A.

Fig. 6.19. PGIEF (pH 4.0 - 6.0) of isolated horse haptoglobulin (Hp) and of sera from 2 horses before (A) and after (B) the development of paralytic ileus.


Hp
A
B
A
B

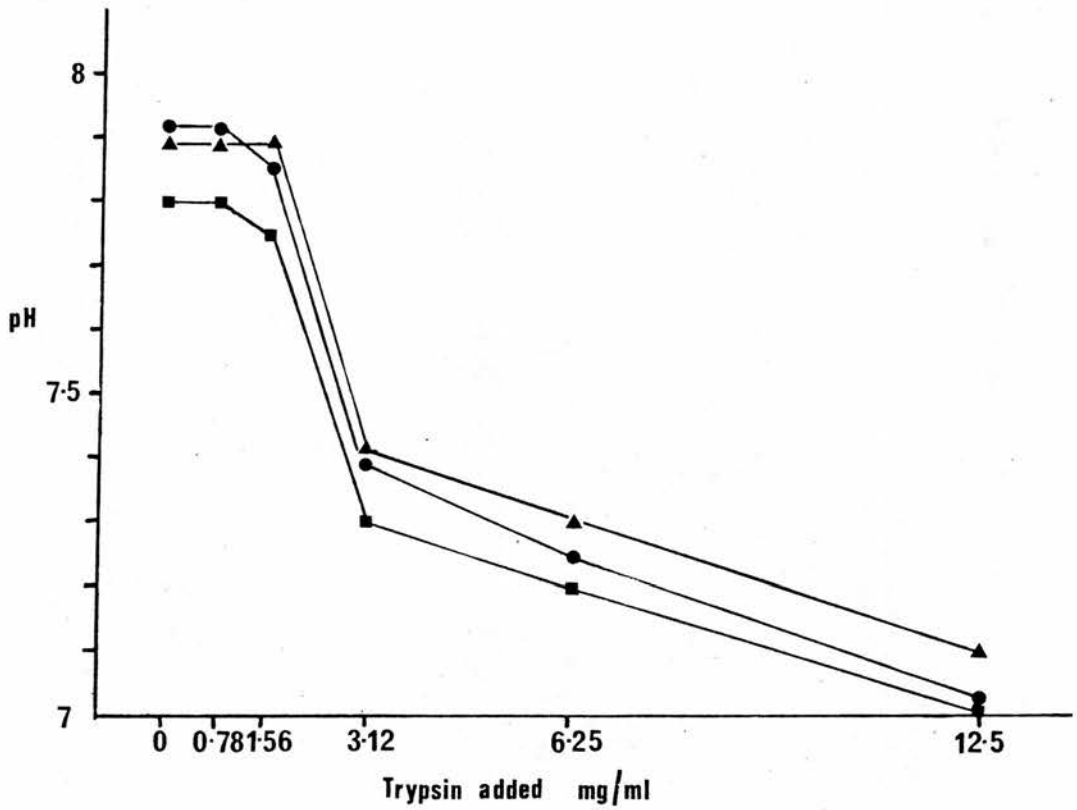


Fig. 6.20 Change in pH of 3 sera following incubation with increasing amounts of bovine pancreatic trypsin.

■ - Serum a (Fig. 6.21)

▲ - Serum b (Fig. 6.21)

● - Serum c (Fig. 6.21)

after PGIEF (pH 4.0 - 6.0) is shown in Figure 6.28. The pI range of these isozymes is 4.36 - 4.74. The more acidic pI corresponds to that of the most anodal band of the electrophoretically fastest EsF allele product (Fig. 6.28).

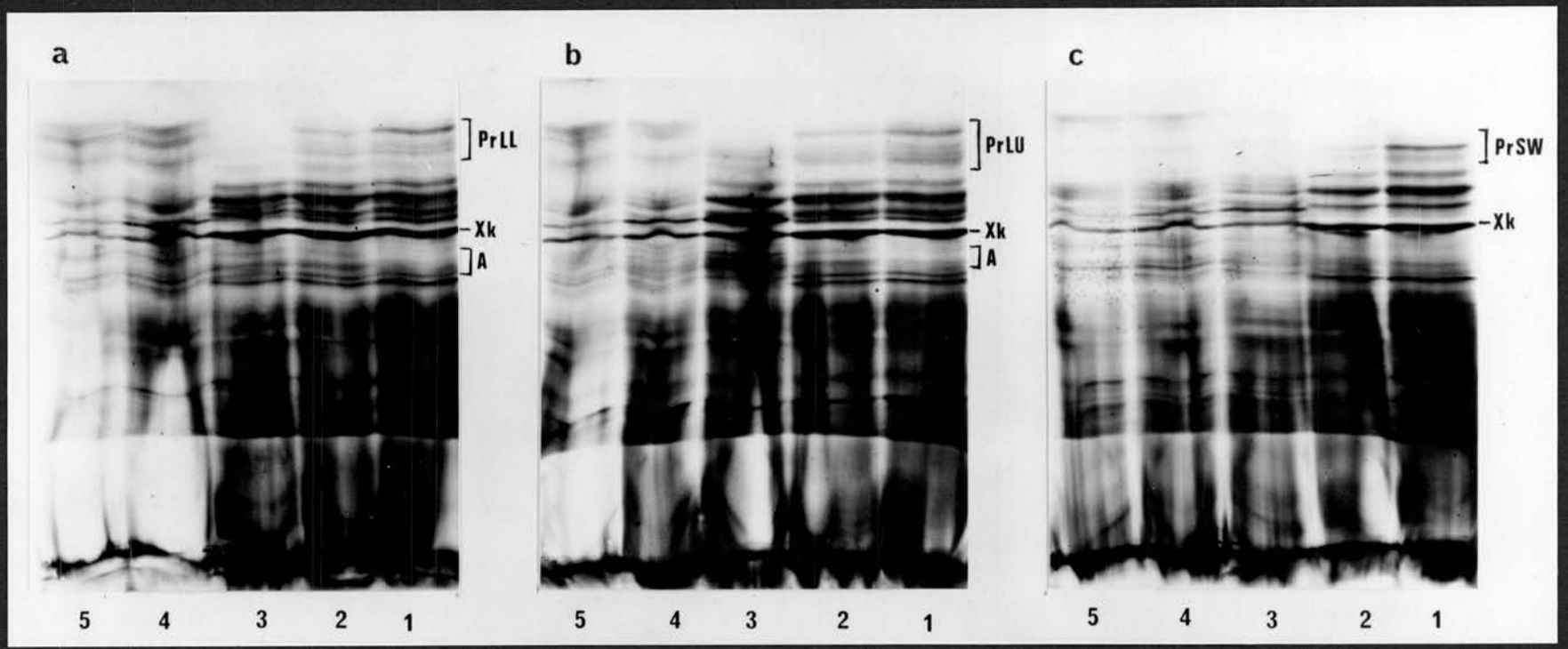
Pr Antiprotease: The post incubation pH of the serum-trypsin mixtures is shown in Figure 6.20. The fall in pH between 0.78mg and 3.12mg enzyme per ml serum indicates saturation of the serum antiprotease mechanisms. The fall in pH is due to hydrogen ion liberation during dissociation of the terminal amino groups of the polypeptide fragments after hydrolysis of serum proteins by free trypsin.

The appearance of the prealbumin bands after incubation of serum with trypsin is shown in Figure 6.21. After the addition of 0.78mg trypsin per ml of serum, there is a reduction in staining intensity of the most anodal prealbumin bands corresponding to the Pr protein. The remaining bands are unchanged, although in sera(a) and (b) new bands appear cathodal to Xk in the region marked A.

After the addition of 1.56 mg/ml of trypsin the Pr bands disappear in all 3 sera, to be replaced by new bands in serum (b). In serum (c) there is also disruption of the remaining bands similar to that resulting from higher trypsin concentrations, indicating that it is probably due to non-specific serum protein hydrolysis by excess enzyme. After the addition of

Fig. 6.21. PGIEF (pH 4.0 - 6.0) of 3 horse sera, (a), (b) and (c), following incubation with increasing amounts of bovine pancreatic trypsin.

1. Serum/PBS control.
2. 0.78 mg/ml trypsin added.
3. 1.56mg/ml trypsin added.
4. 3.12mg/ml trypsin added.
5. 6.25mg/ml trypsin added.


a
PrLL
Xk
A
5
4
3
2
1
b
PrLU
Xk
A
5
4
3
2
1
c
PrSW
Xk
5
4
3
2
1

3.12mg/ml and 6.25mg/ml of trypsin there is marked disruption of the prealbumin bands in all 3 sera. New bands are apparent in the Pr region whose configuration resembles that of the PrLL phenotypes. However new bands are the same in all 3 sera suggesting that they are due to non-specific proteolysis. These observations show that protease binding is restricted to the more anodal prealbumin groups which correspond to the Pr protein. The pI of the Pr protein variants varies from 4.04 (Pr F product) to 4.36 (Pr W product).

(d) Discussion: The 3 major acidic prealbumin proteins identified after ASGE (pH 4.3) of horse serum have been identified after PGIEF (pH 4.0 - 6.0). The relative distribution of these proteins is similar using both techniques. The distribution of these proteins and the Xk protein after PGIEF is shown schematically in Figure 6.22.

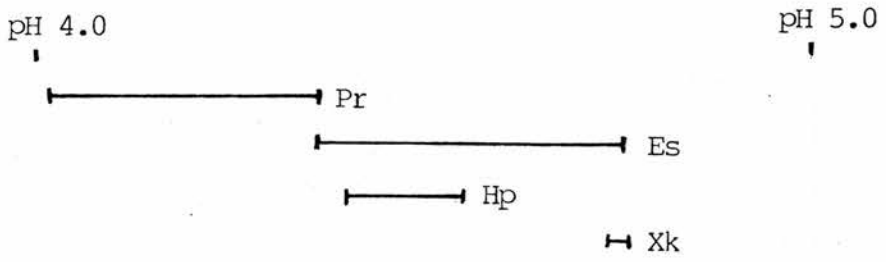


Fig. 6.22. Schematic drawing of the relative distribution of the 3 major acid prealbumin proteins of horse serum after PGIEF (pH 4.0 - 6.0).

Es - aliesterases Hp - Haptoglobin.

The Pr protein appears as a discrete group of bands in the anodal region of the gel. There is overlap of the cathodal PrW product with the most anodal EsF product. With this exception, the discrete nature of the Pr system after PGIEF allows its use in the identification of serum Pr phenotypes. There is no evidence that any prealbumin proteins cathodal to the Pr system can combine with trypsin when free Pr protein is available.

The distribution of the two remaining acidic prealbumin proteins identified after ASGE (pH 4.3) in relation to the distribution of proteins after PGIEF is now known. However as previously discussed, one of these proteins may correspond to the acidic boundary protein. The second may correspond to a polymorphic protein which appears to overlap both haptoglobin and the aliesterases, with a pI around 4.45 to 4.60. The occurrence of such a protein is suggested by the wide variation encountered in this region between individual sera (Figs. 6.24 and 6.25).

(v) Pr phenotyping using PGIEF (pH 4.0 - 6.0).

The technique of thin layer polyacrylamide gel electrofocusing of horse serum in an acidic gradient (pH 4.0 - 6.0) and the application of the technique to Pr phenotyping has been published (Matthews, 1979).

After PGIEF (pH 4.0 - 6.0) the appearance of the Pr variants is basically similar to that on starch gels. However, the number and resolution of the bands in the

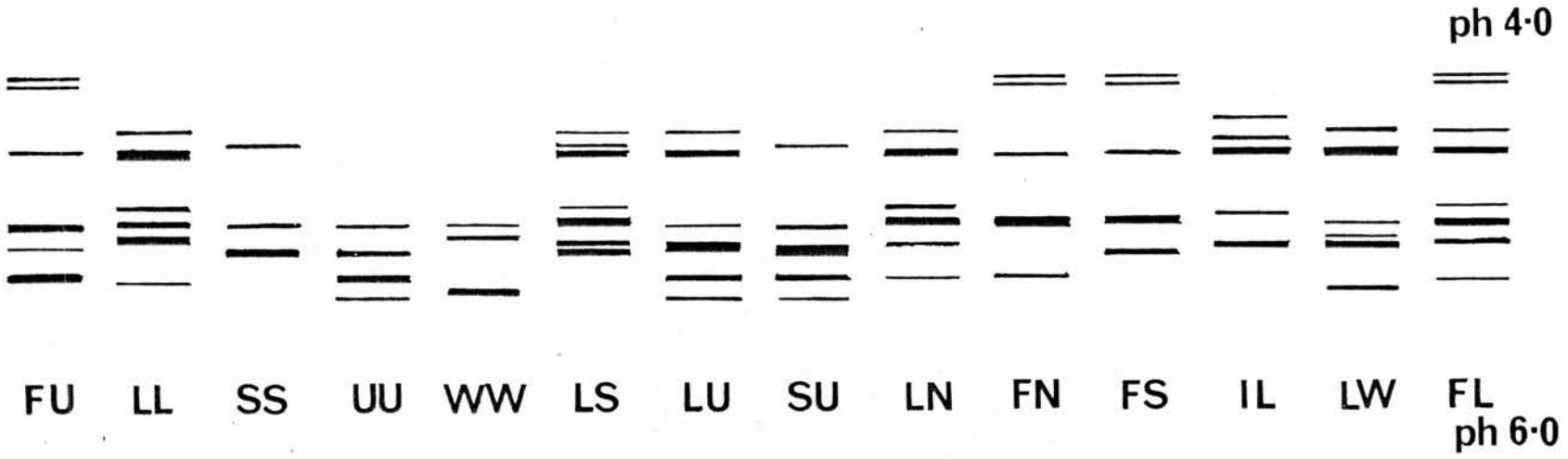


Fig. 6.23. Schematic appearance of Pr phenotypes after PGIEF (pH 4.0 - 6.0).

Fig. 6.24. PGIEF (pH 4.0 - 6.0) of horse serum, showing the Pr phenotypes of the individual sera.

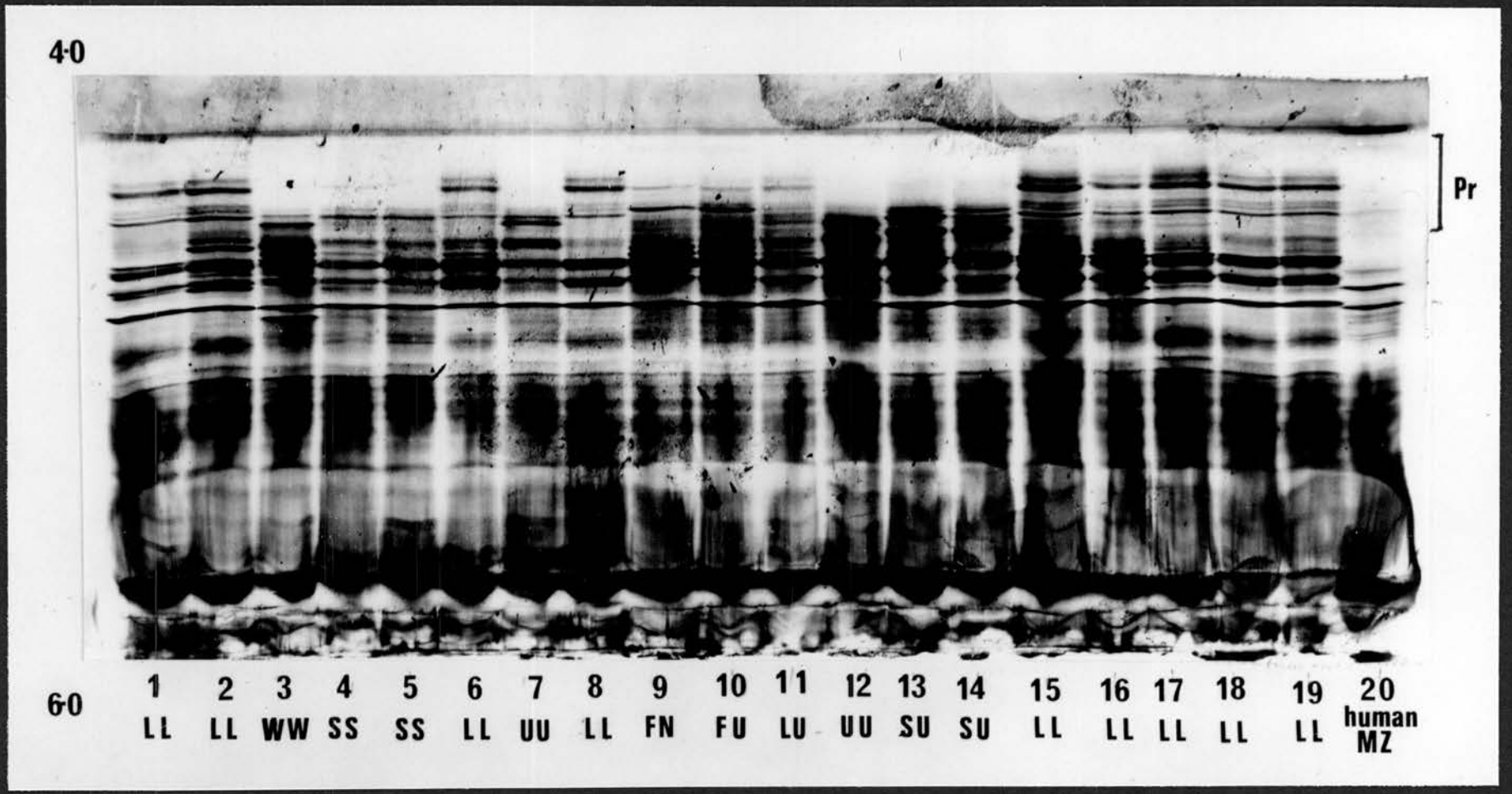
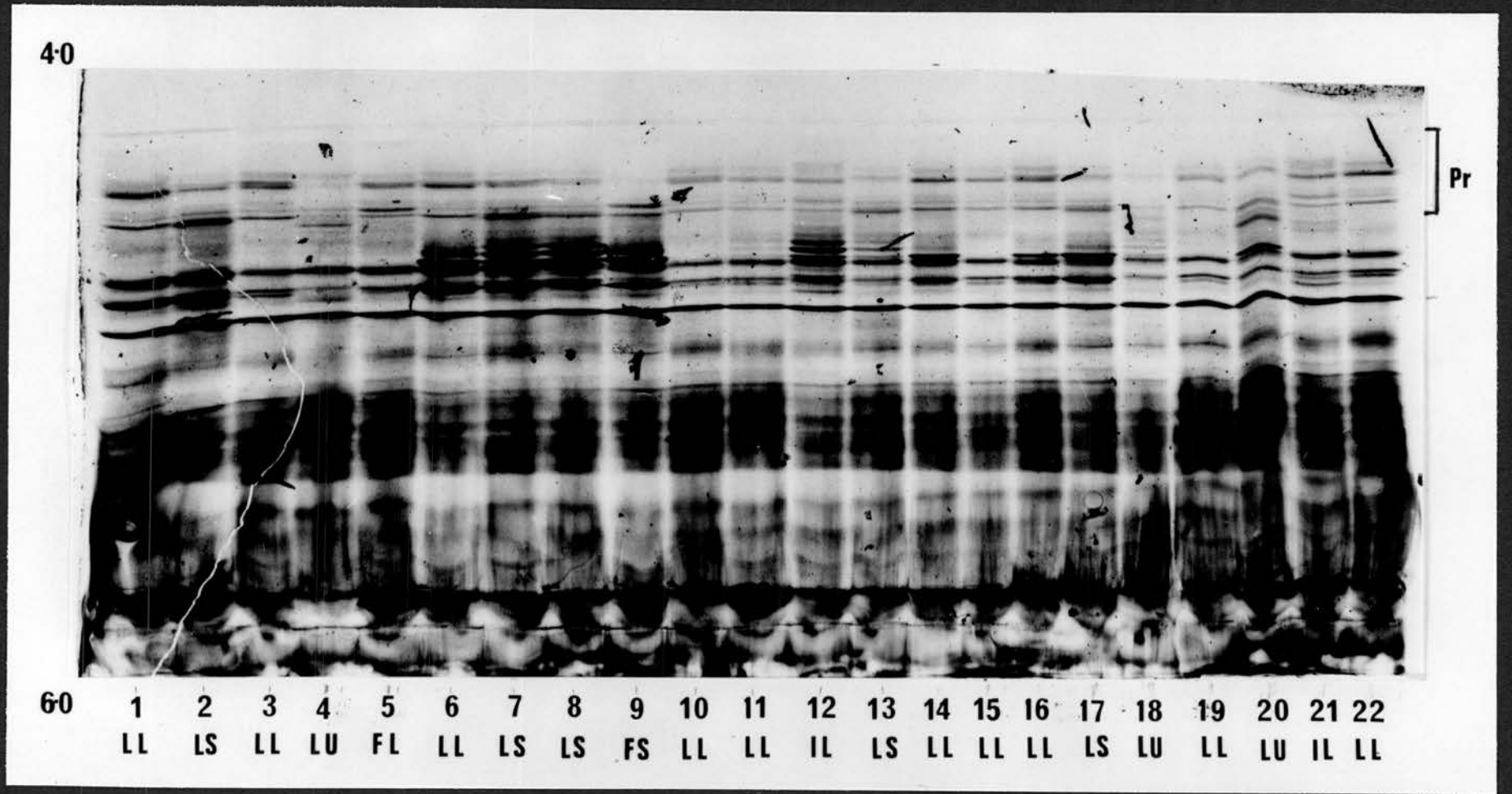

4·0
Pr
6·0
1 LL
2 LL
3 WW
4 SS
5 SS
6 LL
7 UU
8 LL
9 FN
10 FU
11 LU
12 UU
13 SU
14 SU
15 LL
16 LL
17 LL
18 LL
19 LL
20 human MZ

Fig. 6.25. PGIEF (pH 4.0 - 6.0) of horse serum, showing the Pr phenotypes of the individual sera.


4·0
Pr
6·0
1 LL
2 LS
3 LL
4 LU
5 FL
6 LL
7 LS
8 LS
9 FS
10 LL
11 LL
12 IL
13 LS
14 LL
15 LL
16 LL
17 LS
18 LU
19 LL
20 LU
21 IL
22 LL

Pr system is increased, each allele controlling a multiple band pattern of one or two intensely stained bands, with a variable number of lightly stained bands. These are shown diagramatically in Fig. 6.23. Some variation in the staining intensity and in the number of the minor bands occurs between sera of the same Pr type and the same sera on different gels.

After PGIEF, the PrF allele products, recognized only in heterozygotes in this series, appear as indistinct double bands near the lower pI limit of the protein, with third and fourth bands pI indistinguishable from the major PrL and PrN allele products respectively. These bands are shown in the FN, FU (Fig. 6.24, 9 and 10), FL (Fig. 6.25, 5) and FS (Fig. 6.25,9) phenotypes. The PrI allele products, again recognized only in heterozygotes in this series, appear as 2 zones with pI distributed about that of the most acidic PrL allele products, with an additional bands of pI slightly more acidic than the common PrN and PrF allele product. These bands are shown in the IL phenotype (Fig. 6.25, 12 and 21). In cases where the PrI allele products are weakly expressed, the 2 more acidic zones may be indistinct.

The PrL allele product shows the 3 bands characteristic of the homozygote on acidic starch gel electrophoresis (Braend, 1970). However, 2 additional bands of intermediate pI are apparent in the homozygote (Fig. 6.24, 1 and 2), the pI of the least

acidic of these 2 bands being indistinguishable from the apparently common PrN and PrF bands. In a number of PrL homozygotes, 1 or 2 additional bands, of lower pI than those already described are apparent (Fig. 6.24, 15, 16 and 17). The more intensely staining of the most acidic PrL bands may in some animals be separated into 2 bands of close pI, as shown in an LL type in Fig. 6.25, 3. The less acidic of these two 'sub' bands has a pI close to a variable band considered to be a PrS allele product, shown in the adjacent LS phenotype (Fig. 6.25, 2) and in an SS phenotype (Fig. 6.25, 4). An LS type with only the more acidic of these 'sub' bands is shown in Fig. 6.25, 13.

The PrS allele product appears heterogeneous. In addition to the variable acidic band already described in Fig. 6.25, 2, the PrS allele product also appears to have a major band of varying pI. In some animals, the pI of this band is similar to that of the least acidic of the major PrL bands as seen in the SS type (Fig. 6.24, 4 and 5) and PrLS type (Fig. 6.25, 13) while in others the pI of the S band is of a distinctly less acidic pI than the PrL band, as seen in the SU (Fig. 6.24, 13 and 14) and LS types (Fig. 6.25, 2, 7, 8, 13 and 17). The PrS product has an additional band of pI similar to the common PrF, PrN and PrL bands.

TABLE 6.5

COMPARISON OF THE Pr PHENOTYPES DETERMINED BY PGIEF (pH 4.0-6.0) AND BY (a) ASGE (pH 4.3) AND (b) ASGE (pH 4.8).

(a) Thoroughbreds - Individual animals are arranged in mating groups;

S = Sire D = Dam F = Foal

HORSE	PGIEF	ASGE (pH 4.3)	DISCREPANCY
S_1	LU	LU	
D_1	SU	SU	
F	UU	UU	
D_2	FL	FL	
F	LU	LU	
D_3	LL	LL	
F	LL	LL	
F	LU	LU	
D_4	LL	LL	
F	LU	LU	
D_5	FL	FL	
F	FU	FU	
S_2	LS	LS	
D_6	LL	LL	
F	LS	LL	
D_7	LS	LS	
F	LL	LL	
D_8	FN	FS	*
F	NS	FS	*
D_9	LS	LS	
F	LS	LS	
S_3	LS	LS	
D_{10}	LL	LL	
F	LL	LL	
D_{11}	LL	LL	
F	LS	LS	

Continued/...

TABLE 6.5. (continued).

HORSE	PGIEF	ASGE (pH 4.3)	DISCREPANCY
S_4	LS	LS	
D_{12}	LL	LS	*
F	LL	LL	
D_{13}	SU	SU	
F	SS	SS	
D_{14}	FL	FN	*
F	LL	LN	*
S_5	LL	LL	
D_{15}	LL	LL	
F	LL	LL	
F	LL	LL	
D_{16}	LL	LL	
F	LL	LL	
D_{12}	LL	LS	*
F	LL	LL	
F	LS(atypical L product)	LS	
D_{17}	IL	IL	
F	IL	IL	
F	LL	LL	
F	IL	IL	
D_{18}	LU	LU	
F	LU	LU	
F	LU	LU	
S_6	LU	LU	
D_{19}	LS	LS	
F	LL	LL	
F	SU	SU	
D_{20}	SU	SU(Weak S band)	
F	UU	UU	
F	SU	SU	
D_{21}	IL	IL	
F	LL(atypical L product)	LL	
F	IL	IL	

CONTINUED/...

TABLE 6.5. (Continued).

HORSE	PGIEF	ASGE (pH 4.3)	DISCREPANCY
D_{22}	FL	FL	
F	FU	FU	
D_{23}**	FN	FU	*
F	FN	FN? (Weakly expressed)	

** Sire - part T.B.; Pr type LN.

(b) Mixed bred population

HORSE	PGIEF	ASGE (pH 4.8) (Braend,1970)	Discrepancy
1	IS	IS	
2	LU	LW (atypical W product)	
3	IL	IL	
4	LU	LN? (No N product when checked)	
5	FL	FL	
6	LL	LL	
7	LS	LL possibly LS	
8	LW	LW	
9	FS	FS	
10	LL	LL	
11	LL	LL	
12	IS	IS	
13	LS	LS	
14	SS	FS	*
15	NU	FL	*
16	NS	NS	
17	LL	NS	*
18	NS	NS	
19	NU(atypical)	IS	*
20	FN	FL	*
21	SU	SU(atypical U product)	
22	SS	IS (weak I product)	
23	LL	LL	
24	LN	IN	*
25	LN	LN	
26	SS	IS	
27	IL	IL	
28	IS	?	
29	LS	LS	
30	FL	FL	

The PrU allele product (Fig. 6.24, 7 and 12) appears as 2 distinct bands, the more acidic having a pI similar to the major S band. In some cases, 2 additional bands of more and less acidic pI's respectively are apparent.

The protein bands considered to represent the PrW allele products are shown in Fig. 6.24, 3 in a sample taken from a non-Thoroughbred animal.

Comparison of the Pr phenotypes within a family based population of TB horses determined both by PGIEF and ASGE (pH 4.3) are presented in Table 6.5a. Similarly comparison of the Pr phenotypes in a population of mixed breeding determined by the present author and by Dr. Braend using PGIEF and ASGE (pH 4.8) respectively are shown in Table 6.5b. Comparison of both Pr phenotyping methods show that discrepancies occur principally in the diagnosis of the Pr F, I, and N allele products after PGIEF. This may result from the relative difficulty in distinguishing the former two variants after PGIEF. The N variant, whose major product shares a common pI with minor bands of the Pr E L, S and U alleles, presents difficulty in differentiation, particularly from the heterozygous forms of these overlapping allele products.

(vi) Discussion

In comparison with ASGE, the PGIEF technique results in increased and consistent resolution of prealbumin protein zones, although the ensuing complexity of the

banding patterns may cause difficulty in interpretation. The technique however is free of the technical difficulties of the ASGE method. It was found that a 6 hour electrofocusing period is necessary to achieve separation of the prealbumin zones comparable to that achieved using ASGE. Fisher and Scott (1978) were able to determine the phenotypes of serum aliesterase isozymes after a 2 hour focusing period on sucrose-free 5% ($^{w}/v$) polyacrylamide gel using a 3.5 - 5.0 pH gradient, in contrast to the 6.5% ($^{w}/v$) polyacrylamide gel used in the present study. However the relatively greater molecular sieving effects of the latter gel may account for the increased focusing time required (Righetti and Drysdale, 1976).

The occurrence of multiple protein species in the inter Pr-Xk region after both ASGE and PGIEF is supported by the recent observations of Juneja, Gahne and Sandberg (1979). Using two dimensional electrophoresis with first dimension separation in 1% agarose (pH 5.4) these authors reported at least 5 electrophoretically distinct protein types in the prealbumin region at acidic pH.

The protease probe method has shown that after PGIEF only the more anodal bands are able to complex with the enzyme. Ek (1977) has demonstrated the removal of the Pr bands after ASGE (pH 4.8) by the

addition of increasing amounts of bovine trypsin. As in the present study, this author failed to demonstrate alteration in banding pattern within the inter Pr-Xk zone except in the presence of excess enzyme. Ek (1977) suggested that new bands appearing in the inter Pr-Xk zone in the presence of excess trypsin may in part be inhibitor-enzyme complexes. However, the association of new bands with inhibitor-enzyme complexes can be considered only in the presence of excess inhibitor, as in the case of the new bands (A) shown in Fig. 6.21, (a) and (b). The pI difference between the postulated Pr-enzyme complexes (A) after PGIEF and the native Pr protein is approximately 0.6 - 0.7 which compares with the 0.5 - 0.8 difference between the free Pi inhibitor and inhibitor-enzyme complexes in human serum (Saklatvala et al., 1976).

The protease probe technique demonstrated antiprotease activity associated with all the bands within the Pr zone. However (Scott, 1980; pers. comm.) has shown that the Rf of the most anodal of the PrG allele products on ASGE does not alter following the addition of trypsin and Pollitt and Bell (1980) also failed to demonstrate antiprotease activity with this band using the chromatic, ester substrate staining method of Uriel and Berges. Such observations do not exclude this protein from the Pr antiprotease system since Gahne's (1966) results on neuraminidase treatment of horse serum suggest that

the electrophoretic heterogeneity of the Pr allele products appear determined principally by variation in the amino acid sequence of the polypeptide chain. Thus primary structure variation in the most anodal PrG product may sterically or functionally inhibit the enzyme binding site.

While the respective mobilities of the major products of the individual Pr alleles after PGIEF (pH 4.0 - 6.0) are essentially similar to those described by Braend (1970) after ASGE (pH 4.8), in some cases the number of minor bands is increased. However, examination of the crossed electrophoretic patterns of Ek (1979) (e.g. Fig. 6.11) show that within the presently defined Pr system after ASGE (pH 4.8) additional allele products do occur but are inapparent using routine starch gel techniques. Furthermore, although the individual homozygote patterns shown by Ek (1979) are not directly comparable due to intergel variation in separation, a band appears in the Pr F, L, S and U homozygotes after ASGE (pH 4.8) which has a mobility comparable to that of the major Pr N product. A common band amongst these phenotypes was observed after PGIEF.

Comparison of the PGIEF products with those after ASGE (pH 4.3) show differences principally in the mobilities of the major N and S products, these being relatively slower than on starch gels. However

the relative mobility of these products after PGIEF is similar to that after ASGE (pH 4.8) (Braend, 1970), which may reflect a unique pH dependent mobility of these products, as has been previously suggested (6.6.1(iii)). Differences in the appearance of the Pr variants demonstrable using PGIEF and ASGE at both pH 4.3 and pH 4.8 are reflected in the Pr phenotype diagnosis shown in Table 6.5, a and b respectively. The confusion resulting from the similarity of the major N and L products after ASGE (pH 4.3) (6.6.1.(iii)) is shown in Table 6.5a, D14 and offspring, where no N product was demonstrable in either case on PGIEF following its earlier identification on ASGE. Conversely, the overlap of the more acidic of the major PrS band variants after PGIEF with the minor least acidic PrL band resulted in the diagnosis of a PrLL type in the case of D_{12} (Table 6.5, a) whose serum contained a typical S band after ASGE (pH 4.8). However, the overlap of the major PrN band after PGIEF with minor bands of all other alleles except PrI is probably the most important drawback to the use of this technique for routine Pr phenotyping. Discrepancies resulting from this overlap are shown in Table 6.5a, D8 and offspring, and in Table 6.5b, numbers 14 and 15. In the latter case the difficulty in distinguishing the most acidic PrF products after PGIEF compound the difficulties in

Pr diagnosis. The occasional difficulty in recognition of the more acidic PrI zones after PGIEF can also result in incorrect Pr identification (Table 6.5, b, numbers 22, 24 and 26). The remaining discrepancies between the PGIEF and ASGE determined Pr phenotypes (Table 6.5b, numbers 15, 17 and 19) cannot be so readily explained, although they may result from poor band resolution using ASGE. Despite the drawbacks associated with the multiple banding and overlapping of the Pr products after PGIEF, the reproducibility and resolution achieved using this technique makes it an alternative to ASGE for Pr phenotyping. However, restricting the pH gradient to 4.0 - 6.0 causes difficulty in PrF identification, although lowering the pH gradient may permit better resolution of these bands.

Comparison of the observed and presumed homozygous Pr phenotypes after PGIEF, with those determined by 10% polyacrylamide gel electrophoresis (PAGE) at pH 4.2 - 4.3 by Pollitt and Bell (1980) are shown in Figure 6.26. The number of bands in each phenotype determined using both methods is similar though the prominant, most anodal or acidic bands in the F and N types do not appear after PGIEF. The differences are probably associated with the molecular sieving effect of polyacrylamide gel electrophoresis. The PGIEF method is however simpler and quicker than PAGE

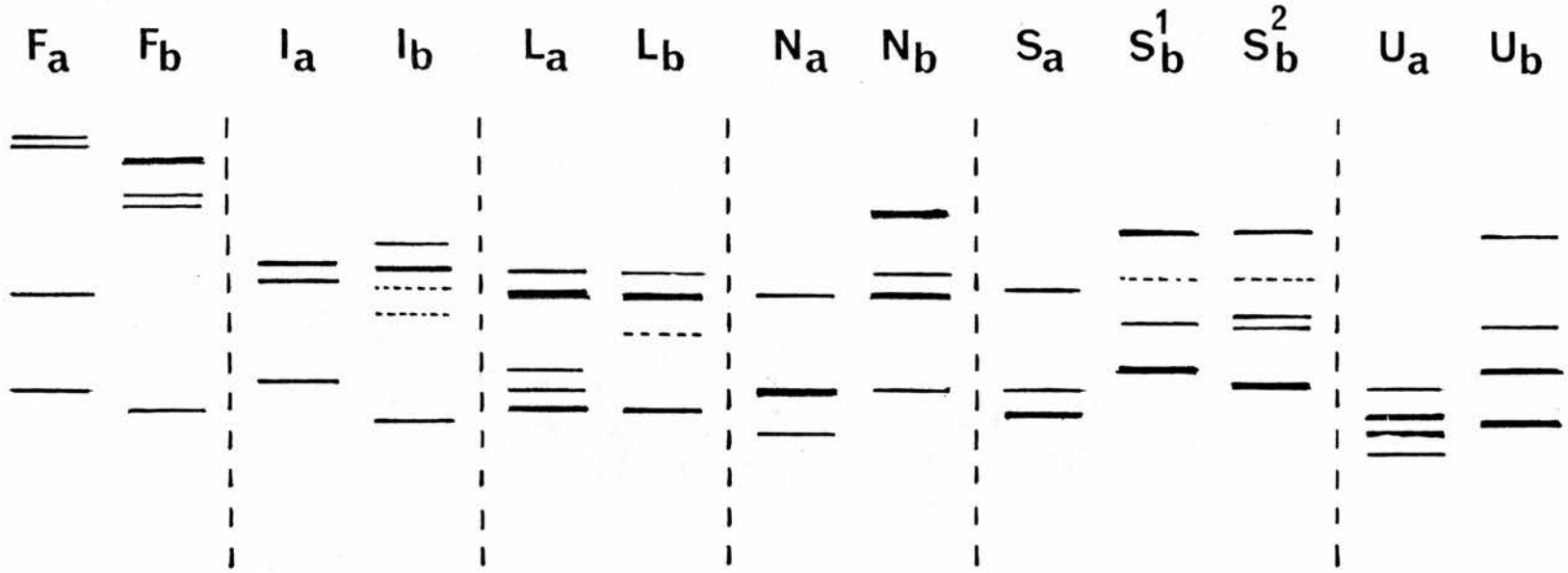


Fig. 6.26. Comparison of the Pr homozygous phenotypes after PGIEF (pH 4.0 - 6.0) and PAGE (pH 4.2 - 4.3) (Pollitt and Bell, 1980)

(a) PGIEF

(b) PAGE, using this system 2 S allele products (S_1 and S_2) were recognised.

though the separation achieved with the latter method is more satisfactory for Pr phenotype diagnosis.

Using PGIEF, previously unrecorded variants of the major PrL and PrS bands have been observed. The double appearance of the major PrL band may represent the genetically determined variation in the L zones alluded to by Braend and Storset (1979; pers. comm.). However in the two family groups in which this variant was observed, no inherited pattern was apparent. This is shown in Fig. 6.25 in which serum 13 is from the offspring of a normal PrLS dam (Fig. 6.25, 14) and a normal PrLL sire indicating that the L variant in the offspring is unlikely to be inherited. Similarly with the PrLS animal whose serum is shown in Fig. 6.25, 2 is the offspring of a PrLL dam (Fig. 6.25, 3) which possesses the double L variants and a normal sire, indicating by the absence of the double L variants from the offspring that the anomaly in this case is not inherited. The cause of this PrL anomaly is unknown although it may result from degeneration of the serum sample. Both bacterial contamination and prolonged storage at room temperature are known to result in additional, anomalous alpha-1 antitrypsin bands during phenotyping of human serum (Lieberman, Kaneshiro and Gaidulus, 1975; Cook, 1975). In addition, where the anomaly was observed in foals, it may have arisen as an age effect. However further family studies of this PrL variant are necessary to establish its origin.

The PrS allele product in this series appears heterogenous. This heterogeneity may correspond to the PrT allele product described by Braend (1970) or to the PrS_1 and S_2 products described by Pollitt and Bell (1980). However, where family data on the PrS variant is available there is no evidence of an inherited pattern.

The PrW allele, whose product after PGIEF differs slightly from that after ASGE (pH 4.8) (Braend, 1970) does not occur in the English Thoroughbred (Scott, 1976) and was not observed in Thoroughbreds in this study. The difference in appearance of the PrW product after PGIEF and ASGE (pH 4.8) led Braend (1979; pers. comm.) to suggest that it may be a subdivision of the PrU product.

The complexity of the molecular heterogeneity of the Pr protein is highlighted in the 1979 horse blood typing comparison test (Juneja, 1980; pers. comm.) in which considerable disagreement on phenotype diagnosis occurred. The confusion is increased by the recent studies of Juneja et al., (1979) which suggest the heterogeneous Pr antiprotease is the product of two linked loci, whose multiple products may be distinguished only by two dimensional electrophoresis. This concept of twin loci is

considered later in Part 4 of this chapter.

Although both ASGE and PGIEF have their respective merits in Pr phenotyping the disadvantages of both methods prompted the examination of a third method of phenotyping - immunofixation electrophoresis, previously described by Johnson (1976) for alpha-1 antitrypsin phenotyping in man.

6.6.3. IMMUNOFIXATION ELECTROPHORESIS OF HORSE ACIDIC PREALBUMIN PROTEINS

(i) Introduction

Immunofixation involves overlaying the electrophoresis gel with high titred specific antisera, resulting in the formation of local immune complexes with the antigen under study. The technique was first described by Alper and Johnson (1969) as a means of examining the electrophoretic variants of human ceruloplasmin, C'3 and Gc proteins. Subsequently, the technique was used to demonstrate human alpha-1 antitrypsin polymorphism after agarose electrophoresis at both acid and alkaline pH (Ritchie and Smith, 1976; Johnson, 1976). The latter author was able to demonstrate new variants at the locus, undetected using conventional ASGE and PGIEF techniques.

Immunofixation electrophoresis in agarose gel (pH 8.6) was used to examine the heterogeneity of horse serum Pr protein at alkaline pH and to investigate the potential use of this technique for Pr

phenotyping.

(ii) Materials and Methods

(a) Sera: Stored Thoroughbred sera of known Pr phenotypes were used throughout.

(b) Electrophoresis: Electrophoresis in 1% (^{w}/v) agarose 5% (^{w}/v) sucrose gels (pH 8.6) was used as previously described (3.2.1.). However, voltage was applied to the gels for 75 minutes, resulting in the migration of bromophenol blue dye albumin marker of 7cms, the maximum possible distance using commercially prepared agarose plates.

(c) Immunofixation: The preparation of rabbit anti-horse acidic prealbumin serum has been previously described (6.4.3). This serum was used undiluted to achieve the high antibody levels necessary for this technique (Alper and Johnson, 1969), After air drying, the gels were overlaid with 2mls of the antiserum and incubated in a moist chamber for 4 hours at 37°C. To conserve the limited amount of antiserum, in later experiments the appropriate zone of the gel was overlaid with a cellulose acetate strip[1] soaked in antiserum as described by Ritchie and Smith (1976). After incubation the gels were washed overnight in 0.9%

1. Cellogel; Gelman, Michegan, U.S.A.

(^{w}/v) NaCl, dried and stained with 1% Amido Black B as previously described (3.2.1.).

(iii) Results

After immunofixation with anti-horse acidic prealbumin serum, three discrete bands appear in the prealbumin-albumin, alpha-1 and fast alpha-2 zones (Figs. 6.27, a and b). These bands showed cathodally trailing arcs on each edge resulting from the diffusive effects of antiserum collected in the longditudinal troughs in the gel.

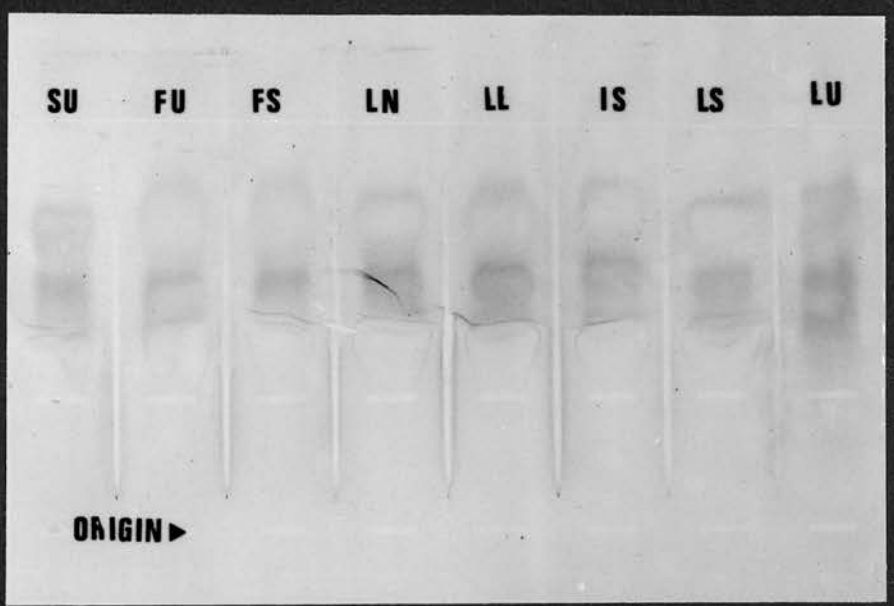
The most anodal band, corresponding to the Pr protein (6.5.), shows marked variation in electrophoretic mobility after prolonged agarose electrophoresis (pH 8.6). Amongst the Pr homozygotes examined, the SS and LL types possessed the fastest and slowest mobility respectively, while the UU adopted an intermediate position (Fig. 6.27, a). Heterozygotes appeared to adopt a mobility corresponding to one or other of their alleles. For example the SU and LU types have a mobility similar to the S and L types respectively (Fig. 6.27, a and b). PrN and PrI allele products were not identified in the series, as homozygous sera was not available. Although Pr FF sera was unavailable, the heterozygous FL, FN and FU types all possessed a similar mobility, faster than that of PrSS type, and presumably that of the F allele product.

Fig. 6.27a and b. Immunofixation electrophoresis of horse acidic prealbumins. The most anodal of the three precipitin bands corresponds to the Pr protein. The Pr phenotype of each sera is given.

A - Albumin migration marker.

a

b



Use of the technique to diagnose the Pr types amongst randomly selected sera was unsuccessful, with the exception of the fast PrF allele product which could be diagnosed with some certainty.

(iv) Discussion

As a method of Pr phenotyping, immunofixation has a number of disadvantages, including the requirement of large volumes of antiserum. Furthermore, the degree of resolution achieved, even in the case of homozygous sera, is insufficient to permit definitive phenotyping. It would appear that the sole potential use of this system is in conjunction with PGIEF (pH 4.0 - 6.0) to identify the PrF product.

The anodal position of the homozygotous Pr S and U products in relation to the PrL product after agarose electrophoresis (pH 8.6) differs from that observed after ASGE (pH 4.3) where both products lie cathodally to the major PrL product. This contrasts with the observations on human alpha-1 antitrypsin variants after agarose gel immunofixation where those variants migrating cathodally to the PiM variant after ASGE (pH 4.8) retain this relative mobility after agarose electrophoresis (pH 8.6) (Johnson, 1976). However, variation in the relative mobilities of the major allele products of the aliesterase locus in horse serum at acid and alkaline pH has been reported (Gahne, 1966; Kaminski, 1972). Juneja et al., (1979) have demonstrated variation in the relative

mobilities of the Pr antiprotease allele products at pH 5.4 and pH 9.0 using two dimensional electrophoresis.

This pH dependant variation in relative mobilities of the Pr allele products may support the contention of Juneja et al., (1979) that the acidic prealbumin antiproteases, rather than being products of a single multiallelic locus, are products of two completely linked loci. This hypothesis is considered in more detail in part 4.

PART 4

6.7. DISCUSSION OF ASPECTS OF THE EXPERIMENTAL RESULTS OF THIS STUDY AND THOSE OF OTHER WORKERS.

6.7.1. INTRODUCTION

The results of this study have demonstrated antiprotease activity associated with the Pr protein of horse serum, a highly polymorphic acidic prealbumin which is the probable homologue of human alpha-1 antitrypsin. However, in both the present study and that of Ek (1977) it has been possible to demonstrate antiprotease activity in the region cathodal to the accepted limits of the Pr protein after ASGE of pH 4.3 and at pH 4.8. However, after both PGIEF (pH 4.0 - 6.0) and ASGE (pH 4.8) (Ek, 1977) no bands outwith the accepted Pr bands appear able to bind trypsin in the presence of free Pr inhibitor. As discussed earlier, although Ek (1979) demonstrated Pr cross reactive bands cathodal to the Pr system after antigen-antibody crossed electrophoresis, these bands appear to be non-functional acid dependant dissociation products of the Pr system. These observations suggest that any antiprotease activity cathodal to the Pr system after ASGE may be associated with a functionally independant antiprotease, and antiprotease activity has tentatively been suggested as being associated with the aliesterases of horse serum (6.5.4.). This hypothesis is now examined in more detail.

In addition, the hypothesis of twin loci governing the acid prealbumin antiprotease activity of horse serum (Juneja et al., 1979) is discussed.

6.7.2. THE HYPOTHETICAL ASSOCIATION OF ANTI-PROTEASE ACTIVITY WITH THE ALIESTERASE ISOZYMES OF HORSE SERUM.

(i) Introduction

Aliesterases, more correctly termed carboxy-esterases on the basis of substrate specificity (Dixon and Webb, 1979), are found in the serum of horses, cats and some smaller laboratory mammals, though not in the serum of primates, dogs or farm animals (Augustinsson, 1961). Carboxyesterases have been found in horse liver (Barman, 1969) and in ox and pig liver (Kirsch, 1971), and on the basis of substrate specificity and inhibition by organophosphate compounds they appear similar to serum carboxyester-ases. Liver carboxyesterases in these species possess an esterolytic activity identical to chymo-trypsin (Kirsch, 1971), and inhibition of horse serum carboxyesterases by disopropyl fluorophosphate (Augustinsson, 1961) is indicative of a serine radical at the active site on the enzyme, a property in common with most mammalian endogenous proteases (Dixon and Webb, 1979).

Pollitt and Bell (1980) have shown that horse serum carboxyesterases are glycoproteins. To test the hypothesis that these enzymes may be protease

inhibitors, able to form inhibitor - protease complexes, the effect of trypsin on the isozyme pattern after PGIEF (pH 4.0 - 6.0) and ASGE (pH 4.8) was examined.

(ii) Materials and Methods

Bovine pancreatic trypsin[1] in 0.01M PBS (pH 7.4) was added to aliquots of 4 horse sera in a 1:9 ratio to give a final enzyme concentration of 0.78mg/ml, 3.12mg/ml and 12.48mg/ml, and the mixtures were incubated for 30 minutes at 37°C. Control dilutions of test serum in PBS were similarly prepared. pH controls were also prepared using test sera whose pH was reduced to that of the 12.48mg/ml serum-enzyme mixture, using Sorenson's phosphate buffer. The final pH of the controls and the test sera were determined using a pH-blood gas analyser[2], and are shown in Table 6.6.

Following incubation, the test and control preparation were subjected to PGIEF (pH 4.0-6.0) as previously described (6.6.2). Aliesterases were detected by immersing the gel in 1% ($^{w}/v$)α- napthyl acetate[1] in 50% acetone to which was added 200mg Fast Blue B[1] in 0.05M phosphate buffer (pH 6.8).

The above experiments were repeated using ASGE (4.8) by the method Braend (1970) in the Blood Typing Laboratory in the Veterinary School in Oslo. However, in these instances trypsin was added to serum in a final dilution of only 3.12mg/ml and 12.48mg/ml

1. Sigma Chemical Co., Poole, England.
2. Corning Instruments, U.S.A.

and the final pH was not determined.

In the case of sera prepared for PGIEF the esterase phenotypes were confirmed by Michael Scott of the Equine Research Station, Newmarket.

TABLE 6.6.

FINAL pH OF THE TEST SERUM-ENZYME MIXTURES AND THE SERUM AND pH CONTROLS.

Serum Preparation	SERUM			
	A	B	C	D
+ Trypsin 0.78 mg/ml	7.8	7.8	7.9	7.9
+ Trypsin 3.12 mg/ml	7.3	7.3	7.4	7.4
+ Trypsin 12.48 mg/ml	7.0	7.0	7.1	7.0
+0.01M PBS (pH 7.4)	7.8	7.8	7.9	7.9
+ Sorensons buffer	7.0	7.0	7.0	7.0

(iii) Results

The effect of preincubation with trypsin on the esterase isozyme pattern for both PGIEF and ASGE is shown in Figures 6.28 and 6.29 respectively. Following saturation of the serum antiprotease mechanisms, indicated by the fall in pH between the 0.78 and 3.12 mg/ml dilutions (Table 6.6), there is a uniform decrease in pI of the isozymes amounting to a 'half-step' (Fig. 6.28, sera a, c, and d) or to one and a half steps (Fig. 6.28, serum b). This effect

Fig. 6.28. PGIEF (pH 4.0-6.0) of horse aliesterases phenotypes FF, II, II and FI following incubation with increasing amounts of bovine pancreatic trypsin.
1 - Serum-PBS control; 2 - Serum-Sorensons buffer pH control; 3 - 0.78mg/ml trypsin added; 4 - 3.12mg/ml trypsin added;
5 - 12.48mg/ml trypsin added.

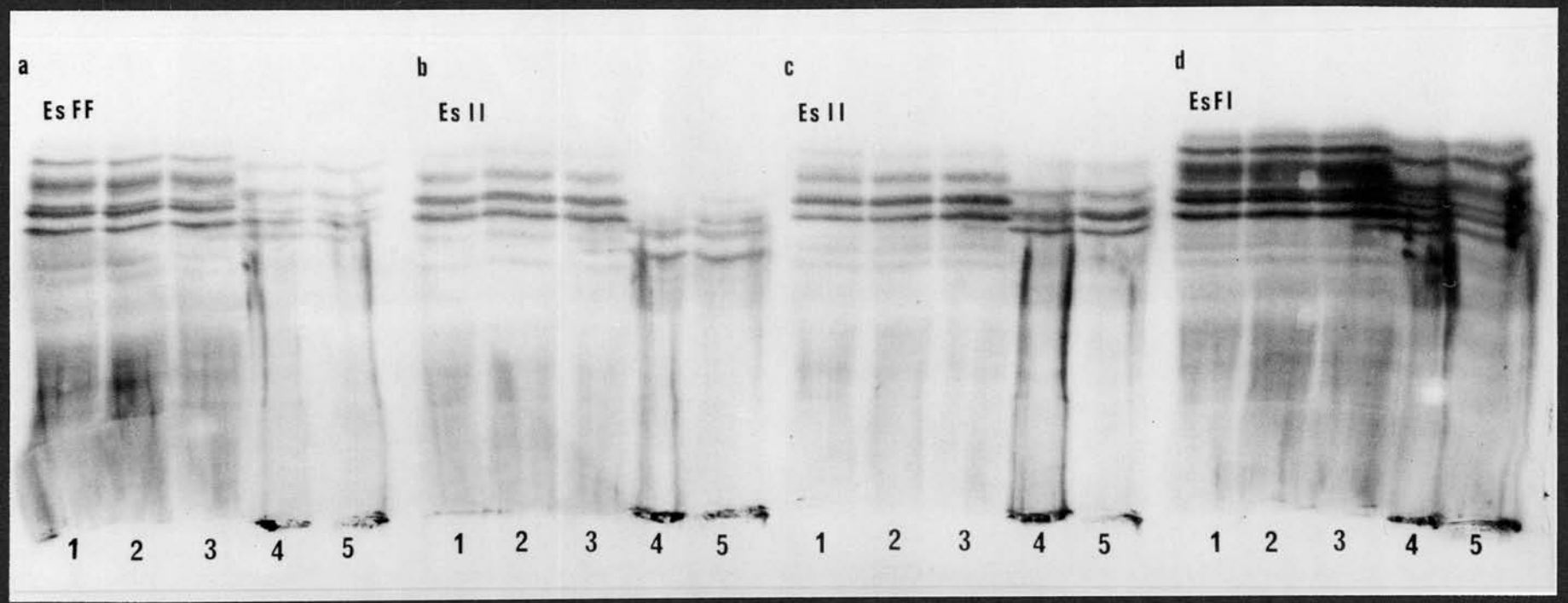
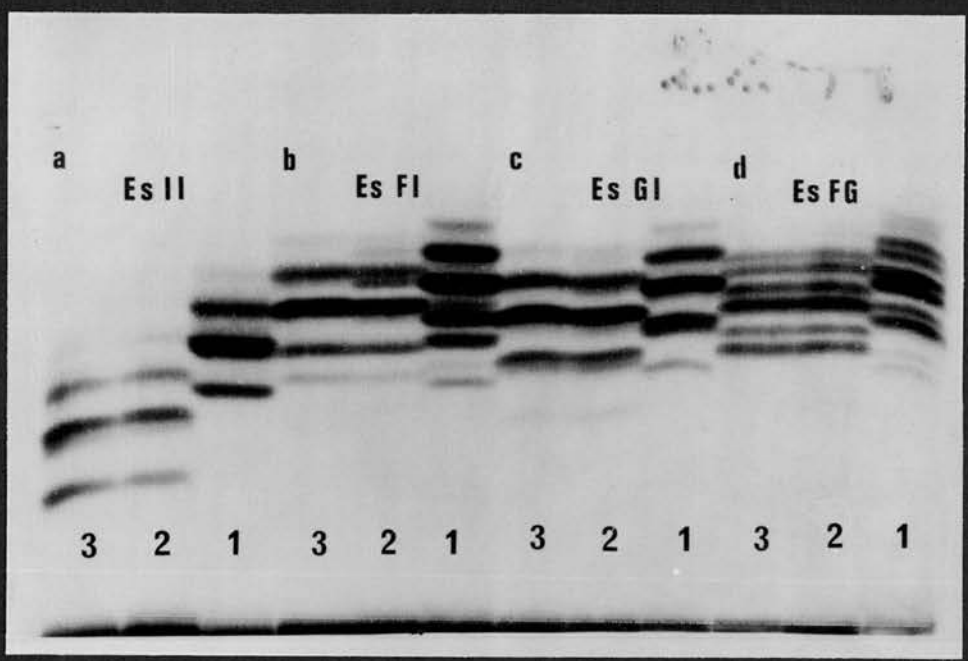

a
Es FF
1 2 3 4 5
b
Es II
1 2 3 4 5
c
Es II
1 2 3 4 5
d
Es FI
1 2 3 4 5

Fig. 6.29. ASGE (pH 4.8) of horse aliesterase phenotypes II, FI, GI and FG following incubation with increased amounts of bovine pancreatic trypsin.
1 - Serum - PBS control; 2 - 3.12mg/ml trypsin added.
3 - 12.48 mg/ml trypsin added.


a
Es II
b
Es FI
c
Es GI
d
Es FG
3 2 1 3 2 1 3 2 1 3 2 1

is independent of pH as shown by the pH controls and is also independent of the amount of enzyme added.

After ASGE (pH 4.8) a similar effect was observed, with a reduction in electrophoretic mobility of either a half or one and a half 'steps' after incubation with trypsin (Fig. 6.29).

After ASGE (Fig. 6.29) the effect of trypsin on the EsI product in the homozygous form differs from that on the EsI product in the heterozygous, EsFI and EsGI forms. However, after PGIEF the two homozygous EsII types shown each adopt different mobilities after incubation with trypsin (Fig. 6.28, sera b and c).

(iv) Discussion

The trypsin induced changes in isozyme mobility on PGIEF and ASGE are similar, indicating the change is the result of alteration in net molecular charge. However, the alteration in mobility differs from that after progressive desialation (Gahne, 1966). Two explanations of the change in isoelectric and electrophoretic mobility on exposure to excess trypsin are possible (Vesterberg, 1973b). The enzyme may either hydrolyse or bind to the polypeptide chain of the esterase, in each case resulting in an decreased net negative molecular charge.

Since the effect of excess trypsin is not progressive, then trypsin hydrolysis of the esterase polypeptide chain can have occurred at only a single

site. Trypsin preferentially hydrolyses polypeptides at bands involving the carboxyl group of L-argenine and L-lysine residues (Dixon and Webb, 1979), and although the amino acid composition of horse serum carboxyesterases is unknown, the liver carboxyesterases in the pig and ox contain between 100 and 140 lysine and argenine residues (Kirsch, 1971). Thus hydrolysis at a single site seems unlikely. However, the two different mobilities of the altered EsI allele products after exposure to trypsin supports the hydrolysis hypothesis, since the 'one and a half step' change could result from the additional loss of a polysaccaride side chain bearing a terminal sialic acid residue. This implies that within the presently defined EsI product two different primary amino acid structures occur, each of which must be the product of separate alleles. This must affect the accuracy of the Es polymorphism in the detection of falsely assigned parentage.

Should the esterases bind trypsin, then the electropositive protease will result in a product of more basic pI and electrophoretic mobility. However, in this case trypsin binding to the esterase will occur only after saturation of the Pr and alpha-2 inhibitors, since the change in Es mobility occurs only in the presence of excess trypsin. Furthermore, the change in Es mobility is not accompanied by any obvious change in its enzymatic activity suggesting that the esterase

activity is independant of any bound trypsin, although the low specificity of trypsin for the substrate napthyl acetate may fail to reveal increased esterase activity due to bound trypsin (Dixon and Webb, 1979). These observations indicate that it is unlikely that the aliesterases are capable of forming inhibitor-enzyme complexes with the protease, and that, unlike human alpha-2 macroglobulin, there is no evidence to suggest that the enzymatic activity of the aliesterases may be associated with bound proteases.

The results of this brief study do not support the hypothetical association of antiprotease activity with horse serum carboxyesterases, and further studies are necessary to determine whether the antiprotease activity apparent in the region cathodal to the Pr system after ASGE is associated with these isozymes or with another acidic prealbumin protein.

6.7.2. ONE OR TWO ACIDIC PREALBUMIN ANTIPROTEASE LOCI IN THE HORSE?

Using two dimensional electrophoresis agarose gel (pH 5.4) followed by polyacrylamide gel (pH 9.0), Juneja *et al.*, (1979) identified two heterogeneous groups of globulins capable of inhibiting the esterolytic activity of both trypsin and chymotrypsin. One group, designated protease inhibitor 1 (Pi1)

showed strong inhibition of both enzymes, while the second group, protease inhibitor 2 (Pi2), though as potent an inhibitor of trypsin as Pi1, showed much weaker chymotrypsin inhibition. Family studies of the extensive polymorphism of both Pi1 and Pi2 proteins indicated that they were controlled by 6 and 5 autosomal codominant alleles respectively but, as no recombinant types were observed, the authors concluded that complete linkage occurred between the two loci. Furthermore a presumed polymeric or aggregate form of the Pi1 protein, but not the Pi2 protein, was demonstrated in normal sera, and Juneja (1980, pers. comm.) has combined Pi1 and Pi2 haplotypes and shown them to equate with observed Pr phenotypes. On this basis these authors proposed that the presently accepted Pr system is the product of two linked loci. In support of this theory these authors cited published data on the isolation of two alpha protease inhibitors, distinct from the macroglobulin, in a number of species (5.6.).

However, Ek (1979) has shown that all the protein bands within the Pr protein system after ASGE share common antigenic determinants and these determinants are also shared with the Pr aggregate (Fig. 6.11). This indicates some degree of molecular homology between the products of the two hypothetical loci.

The hypothesis of twin loci was based in the first instance on the demonstration of functional differences between their products. The method used by Juneja et al., (1979) to demonstrate these functional differences was that of Uriel and Berges (1968) which is based upon the hydrolysis of the protease substrate N-acetyl-DL-phenylalanine B-napthyl ester (APNE). This substrate is readily hydrolysed by chymotrypsin but is only weakly hydrolysed by trypsin (Dixon and Webb, 1979). Thus as Juneja et al. describe antiprotease activity of the Pil and Pi2 proteins as differing only quantitatively, then differences in hydrolytic activity of the two enzymes for APNE could result in the observed differences in inhibitory activity of the two proteins. Should the serum concentration of the hypothetical proposed Pi2 product be less than that of the Pil product, then the much greater activity of chymotrypsin against APNE could effectively 'swamp' the antichymotrypsin activity associated with the Pi2 product, rendering it inapparent, while the antitrypsin activity appears unchanged. To avoid possible misinterpretation of the results the experiments should be repeated using substrates specific for the proteases under examination.

Juneja et al., (1979) also stated that of the two antiprotease loci Pil corresponded to the Pi or alpha-1 antitrypsin locus in human serum. This

proposal was based upon the formation of Pil, but not Pi2 aggregates in horses serum, and in support these authors cited the data of Glaser *et al.* (1977) demonstrating aggregates or polymeric forms of alpha-1 antitrypsin in human serum. However, aggregate forms of human alpha-1 antitrypsin differ from the Pil dimer or aggregate in horse serum. Firstly, alpha-1 antitrypsin aggregates do not occur in normal human serum but are produced only after prolonged incubation of serum in acidic media, and secondly unlike the Pil aggregate human antitrypsin aggregates possess no antiprotease activity (Glaser *et al.*, 1977; Yoshida and Wessels, 1978).

Although the genetic data supports the occurrence of completely linked antiprotease loci, until definite functional differences can be demonstrated between what appear to be antigenically similar groups of proteins, then whether the prealbumin antiproteases are truly the products of two loci remains to be established.

CHAPTER 7

IDENTIFICATION AND CHARACTERISATION OF THE ALPHA-2 ANTIPROTEASE COMPONENT IN HORSE SERUM.

7.1. INTRODUCTION

In man and a number of lower animals the serum alpha-2 antiprotease has been identified as alpha-2 macroglobulin (5.3.2.; 5.6), and Von Fellenburg (1978b) has stated, without the support of experimental observations, that the alpha-2 protease inhibitor observed after fibrinagar electrophoresis of horse serum is alpha-2 macroglobulin. In this chapter the alpha-2 protease inhibitor in horse serum was identified and its electrophoretic heterogeneity and protease binding activity was examined.

7.2. SEPARATION OF THE ALBUMIN ZONE AND ALPHA-2 PROTEASE INHIBITORS OF HORSE SERUM BY GEL FILTRATION CHROMATOGRAPHY.

7.2.1. INTRODUCTION

To separate the two major antiprotease components according to molecular size, horse serum was subjected to gel filtration chromatography and the antitrypsin activity of the serum fractions was determined.

7.2.2. MATERIALS AND METHODS

(i) Sephadex G200 Fractionation

Sephadex G200[1] slurry was prepared in 0.01M PBS (pH 7.4) according to the manufacturers instructions and packed into a 2.5cm x 40cm glass column by gravity flow. 3.5ml of pooled fresh normal horse serum was

applied to the column using an upward flow adaptor and a peristaltic pump giving a flow rate of 27ml/ hour PBS. The protein content of the eluate was monitored on an LKB[1] Uvicord type 4701 with an absorption of ultraviolet light at 280nm and recorded at 10mm per hour on an LKB Chopper Bar Recorder. 7ml fractions were collected using an LKB[1] fraction collection controller type 3403B and those containing protein, fractions 9-20 and 25-30 (Fig. 7.1), were concentrated by evaporation to the original 3.5ml serum volume. The concentrated fractions were dialysed for 24 hours against several changes of PBS.

(iii) Antiprotease activity of Sephadex G200 fractions.

The antitrypsin activity of the individual G200 fractions was determined by fibrinagar electrophoresis as previously described (6.1.1.). The approximate extent of the inhibitory activity associated with each fraction was graded +, ++ and +++.

7.2.3. RESULTS

The protein elution profile of pooled horse serum after Sephadex G200 chromatography is shown in Figure 7.1. The distribution of albumin zone and alpha-2 zone antitrypsin activity in the G200 fractions of horse serum is also presented in Table 7.1.

1. LKB Produckter, Bromma, Sweden.

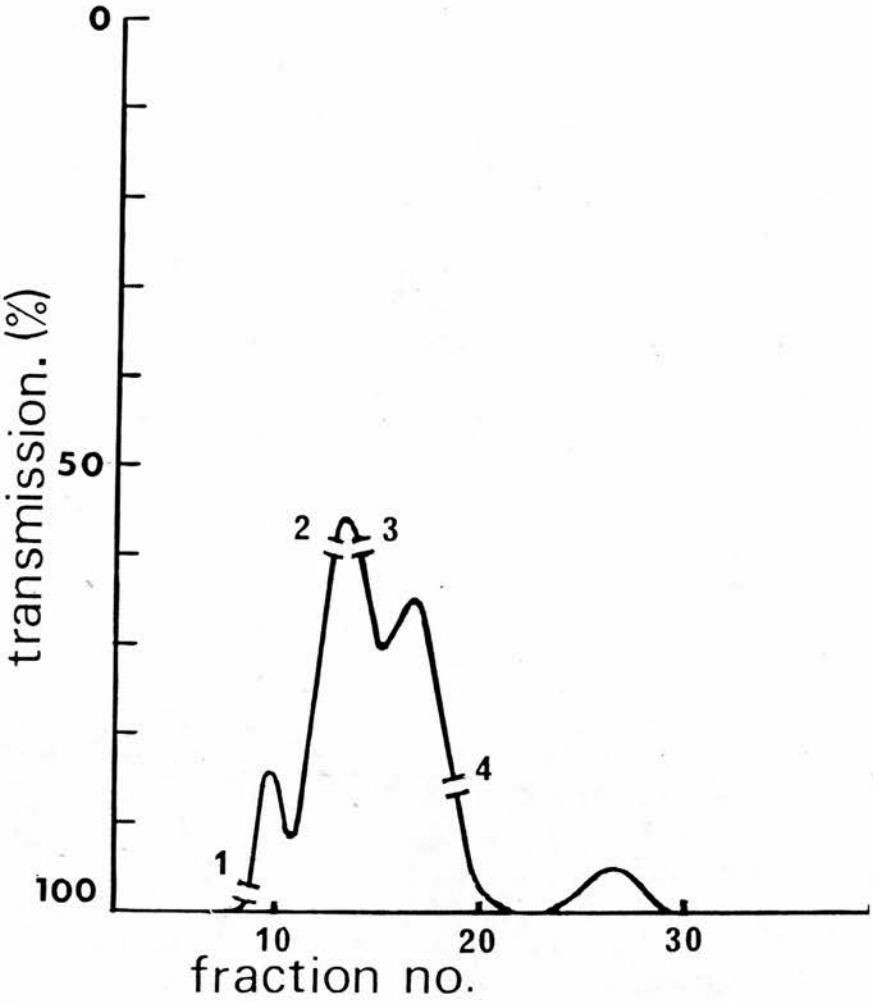


Fig. 7.1. Sephadex G200 chromatography of pooled horse serum showing the distribution of alpha-2 (region 1-2) and albumin zone (region 3-4) antitrypsin activity.

Regions 1-2 and 3-4 on the elution profile (Fig. 7.1) indicate the distribution of the alpha-2 and albumin zone antiprotease activity respectively. The bulk of the alpha-2 activity is eluted with the macroglobulins in the 19S peak and leading edge of the 7S peak, while the albumin zone activity is eluted in the trailing edge of the 7S peak and in the 3S peak.

TABLE 7.1.

ANTITRYPSIN ACTIVITY IN THE SEPHADEX G200 FRACTIONS OF POOLED HORSE SERUM.

Fraction	Alpha-2 Activity	Albumin Zone Activity
9	-	-
10	+++	-
11	++	-
12	++	-
13	+	-
14	-	-
15	-	++
16	-	+++
17	-	+
18	-	++
19	-	+
20	-	-
25-30	-	-

7.2.4. DISCUSSION

The distribution of the alpha-2 and albumin zone antiprotease activity after Sephadex G200 fractionation is similar to that of the alpha-2 and alpha-1 antiproteases in man (Ganrot, 1966a and d).

In addition to carrying the bulk of the alpha-2 antiprotease activity, the 19S peak of horse serum also contains IgM, lipoproteins, alpha-2 macroglobulin and a number of minor proteins (Lavergne and Raynaud, 1970), providing preliminary evidence of an association of the alpha-2 antiprotease with serum alpha-2 macroglobulin.

7.3. IDENTIFICATION OF THE ALPHA-2 ANTIPROTEASE IN HORSE SERUM.

7.3.1. INTRODUCTION

To further examine the association of alpha-2 antiprotease with alpha-2 macroglobulin, the Sephadex G200 fractions of horse serum were subjected to horizontal starch gel electrophoresis using (1) a discontinuous tris-borate buffer system and (2) a

modification of the continuous phosphate buffer system described by Ashton (1960).

7.3.2. MATERIALS AND METHODS

The gel frames, starch preparation and electrophoretic apparatus were identical to those used for acidic starch gel electrophoresis (6.3.2.).

(i) Method I

(a) Gel buffer: 25ml stock solution (8.54g sodium citrate and 17.26g tris hydroxymethylamine per litre distilled water (pH 7.6)) made up to 250ml with distilled water.

(b) Starch: 10% suspension of hydrolysed potato starch[1].

(c) Tank Buffer: 18.55g di-sodium tetraborate and 4g sodium hydroxide made up to 1 litre with distilled water (pH 8.6).

(d) Electrophoresis: Initially electrophoresis was carried out at 6 volts cm^{-1} (120V) for 30 minutes. Following insert removal the voltage was increased 10 volts cm^{-1} (200V) until the borate buffer front had travelled 10cm. The gels were sliced horizontally and stained using 1% nigrosine (w/v) as previously described (6.3.2.).

(ii) Method 2

(a) Gel Buffer: 6mls stock solution (42g di-sodium hydrogen orthophosphate dihydrate per litre distilled water (pH 7.35)) made up to 250ml with distilled water.

1. Sigma Chemical Co., London.

Fig. 7.2. Starch gel electrophoresis (pH 7.6) of whole horse serum and horse serum Sephadex G200 fractions 10-16.

Tf - Transferrins $S\alpha_2$ - Slow alpha-2 globulin

TP? - Thread proteins?

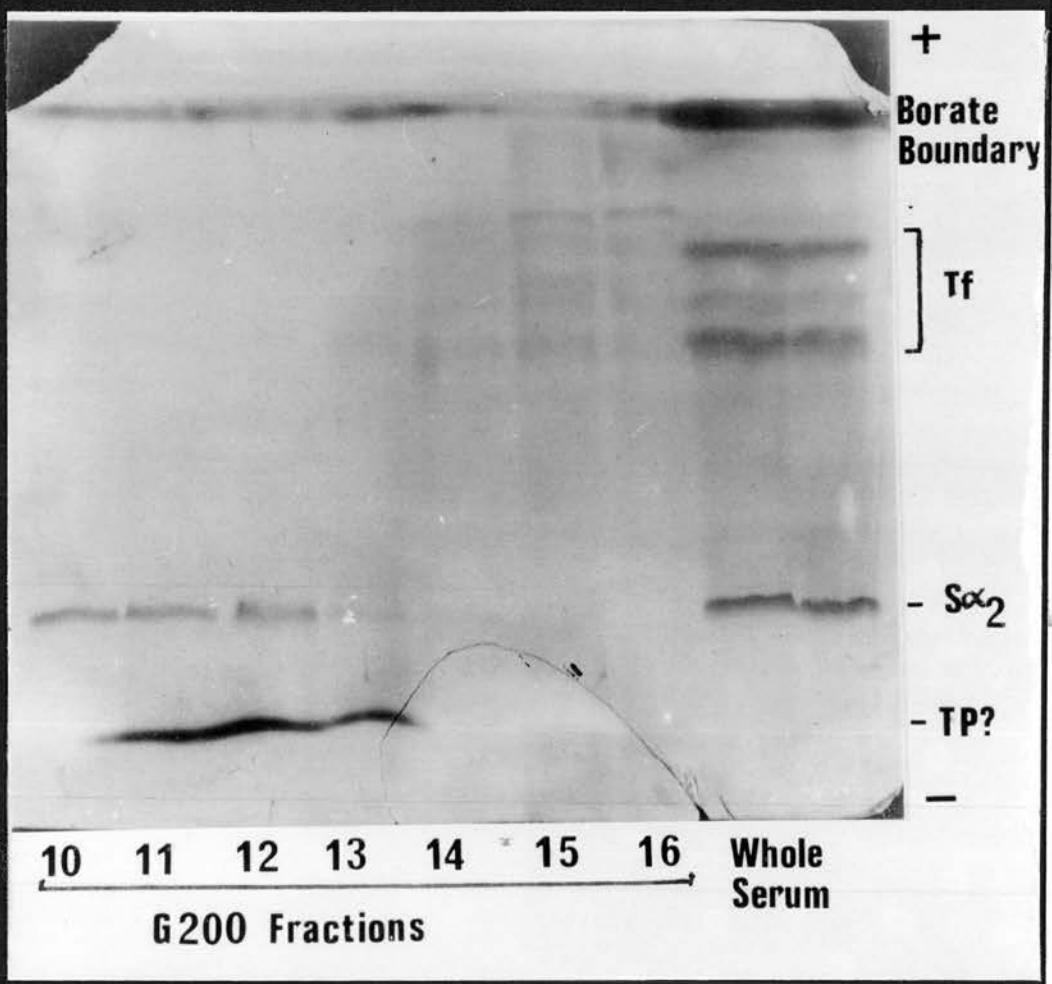
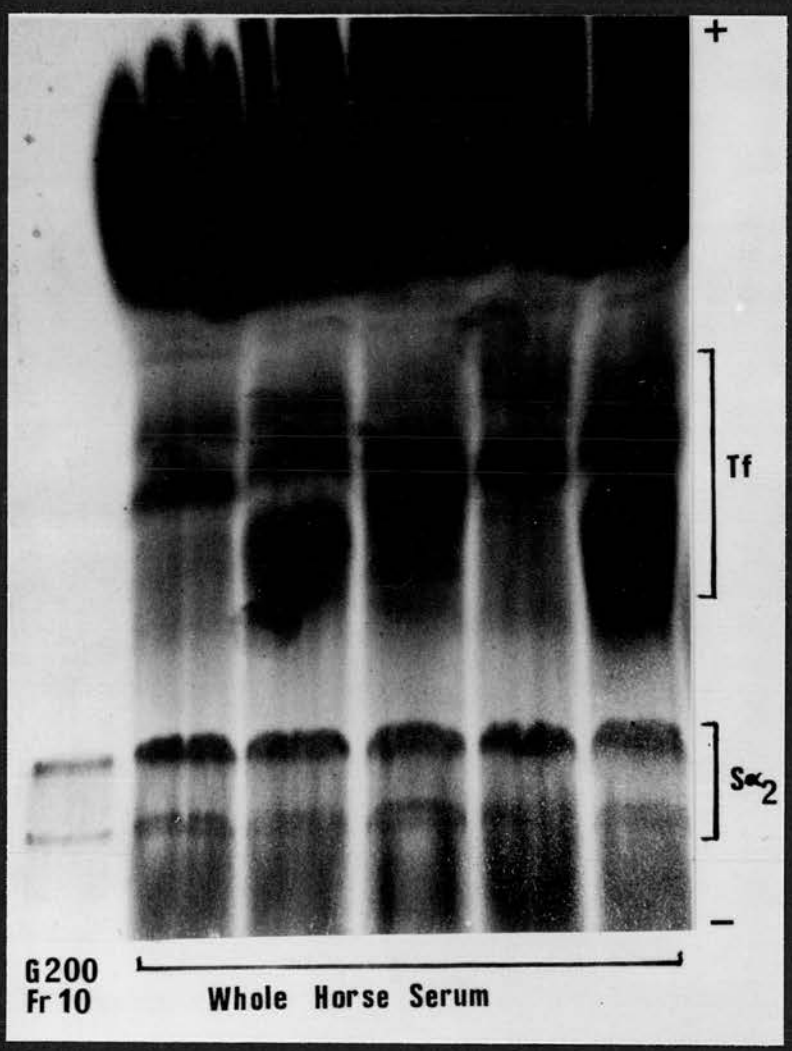

+
Borate
Boundary
Tf
- Sα2
- TP?
—
10 11 12 13 14 15 16
Whole
Serum
G200 Fractions

Fig. 7.3. Starch gel electrophoresis (pH 7.35) of whole horse serum and horse serum Sephadex G200 fraction 10.

Tf - Transferrins

$S\alpha_2$ - Slow alpha-2 globulin.


+
Tf
Sα2
–
G200
Fr 10
Whole Horse Serum

(b) Starch: 13.5% suspension of hydrolysed potato starch[1].

(c) Tank Buffer: As for Method I.

(d) Electrophoresis: Electrophoresis was carried out at 12.5 volts cm^{-2} (250V) for 5 hours. Inserts were removed after 30 minutes. The gels were sliced horizontally and stained using 1% nigrosine (w/v).

7.3.3. RESULTS

Alkaline starch gel electrophoresis (pH 7.6) (method I) of Sephadex G200 fractions 10-16 (Fig. 7.1) and prefractionation pooled horse serum is shown in Fig. 7.2. Fractions 10-13, containing alpha-2 antiprotease activity, contain the slow alpha-2 globulin ($S\alpha_2$) (Ashton, 1958; Gahne, 1966) along with a diffuse group of proteins migrating within the borate boundary zone. The proteins lying cathodally to $S\alpha_2$ in fractions 11-13 are similar in appearance to the so-called thread proteins (TP) (Ashton, 1958). Fractions 14-16, with no alpha-2 antiprotease activity, contain transferrin (Tf) (Gahne, 1966) and the borate boundary proteins, but no $S\alpha_2$.

After starch gel electrophoresis (pH 7.35) (method 2) the $S\alpha_2$ in whole serum is separated into two bands (Fig. 7.3). Similarly, the $S\alpha_2$ globulin

1. Sigma Chemical Co., London.

in G200 fraction 10 may be separated into two components identical to those in whole serum. A discrete anodal boundary was inapparent using a discontinuous phosphate-borate buffer system (pH 7.35).

7.3.4. DISCUSSION

The electrophoretic data shows that the major protein common to those G200 fractions with alpha-2 anitprotease activity (10-13) is the $S\alpha_2$, which after electrophoresis at pH 7.35 is identical to the $S\alpha_2$ in whole serum. The electrophoretic behaviour and molecular size of the $S\alpha_2$ globulin indicates that it corresponds to alpha-2 macroglobulin (Pepper, 1968; Lavergne and Raynaud, 1970).

'Thread proteins' have been described in a number of species although their function is unknown (Ashton, 1958; Schroffel, 1966). However the 'thread protein' like components observed in G200 fractions 11-13 in this series were absent from pre-fractionation whole serum and may be an artefact arising during fractionation and electrophoresis.

7.4. DIRECT DEMONSTRATION OF ANTIPROTEASE ACTIVITY ASSOCIATED WITH HORSE ALPHA-2 MACROGLOBULIN.

7.4.1. INTRODUCTION

The experimental data has so far provided only indirect evidence of an association of alpha-2 antiprotease activity with alpha-2 macroglobulin. To demonstrate a direct association of the antiprotease with the macroglobulin, the fibrinagar inlay and chromatic staining techniques previously used to demonstrate the antiprotease activity of the Pr protein after ASGE (6.5.) were used after alkaline starch gel electrophoresis of horse serum.

7.4.2. MATERIALS AND METHODS

Discontinuous phosphate-borate starch gel electrophoresis (pH 7.35) (7.3.2.) of pooled horse serum was carried out using a single insert strip until the visible albumin zone had migrated 4-6cms. The bottom gel slice was cut into 4 segments along the direction of the run. Three of these segments were stained as follows; for antitrypsin activity using the chromogenic method of Uriel and Berges (1968) (6.4) employing acetyl-DL-phenylalanine β napthyl ester (APNE) as substrate, for esterase activity using the same method without preincubation of the gel with trypsin and for proteins with 1% ($^{w}/v$) nigrosine. In addition a strip was removed from the remaining segment and was incorporated into one of two longditudinal troughs cut in fibrinagar gel as previously

described (6.4.). A 0.032% (w/v) solution of bovine pancreatic trypsin[1] in 0.0025N HCl was added to the remaining trough and the gel incubated in a humidity chamber overnight at 37°C.

7.4.3. RESULTS

The results of fibrinagar-starch gel inlay method (Fig. 7.4, c and d) show a discrete zone of antitrypsin activity (X) corresponding to the double alpha-2 macroglobulin bands. A more diffuse zone of antitrypsin activity (Y) is associated with the more anodal protein mass and overlaps the transferrin and slow albumin zones.

Although the macroglobulin is able to inhibit the fibrinolytic activity of bovine trypsin it possesses only a limited effect on the esterolytic activity of the enzyme, shown by the absence of corresponding zones of negative staining using the chromatic staining technique of Uriel and Berges (Fig. 7.4b). Furthermore, within the alpha-2 macroglobulin region a band of natural esterase activity (Es) is apparent which corresponds to the slower macroglobulin band (Fig. 7.4a). A second, much fainter, esterase band corresponding to the faster alpha-2 macroglobulin band was also observed, though this is inapparent in Figure 7.4a.

The more anodal antitrypsin zones appear able to inhibit both the esterolytic and proteolytic activity of the enzyme, indicated by the zones of negative staining in Figure 7.4b. Some of these antiprotease zones

1. Sigma Chemical Co., London.

Fig. 7.4. Starch gel electrophoresis (pH 7.35) of horse serum.

(a) Chromogenic ester substrate staining for natural esterase activity. Es - indicates band of esterase activity.

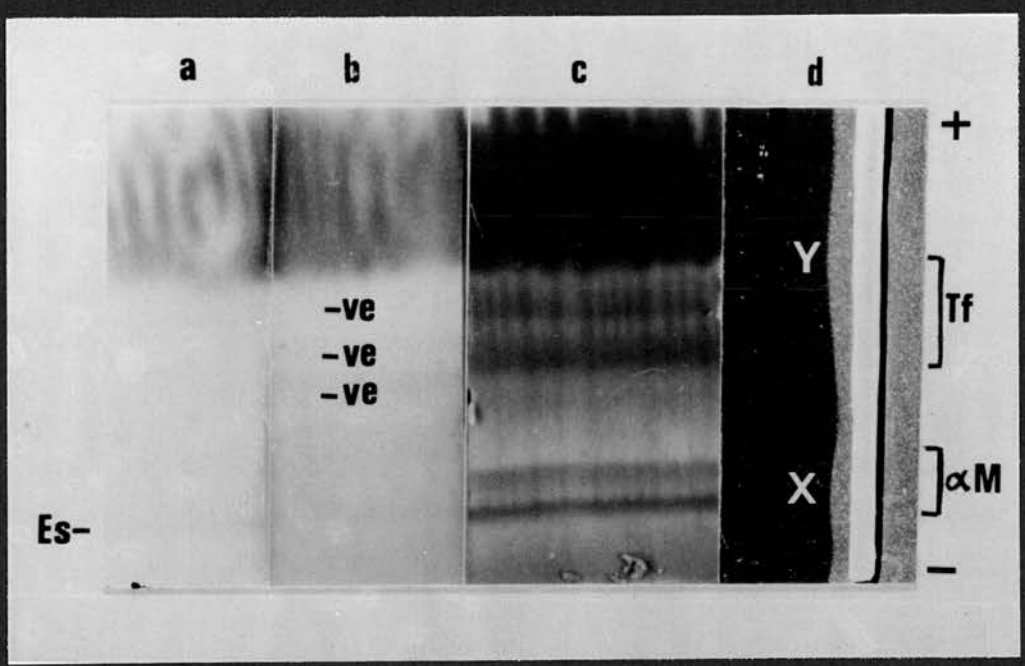
(b) Chromogenic ester substrate staining following incubation of the gel in bovine pancreatic trypsin. -ve indicates zones of inhibition of the esterase activity of trypsin.

(c) Stained 1% nigrosine.

(d) Inhibition of the fibrinolytic activity of trypsin. Left hand trough contains bovine pancreatic trypsin.

Tf - Transferrins

αM - Alpha-2 macroglobulin.


a
b
c
d
+
Y
Tf
-ve
-ve
-ve
αM
X
Es-
–

probably correspond to the Pr protein (Gahne, 1966).

7.4.4. DISCUSSION

The experimental results show that as in other species the electrophoretically slower or alpha-2 antiprotease activity resides in the alpha-2 macroglobulin fraction. The macroglobulin appears able to inhibit the ability of the enzyme to hydrolyse large molecular weight substrates, in this case fibrin, though not small molecular weight substrates, in this case the ester APNE. This property is shared with human alpha-2 macroglobulin (Barrett and Starkey, 1973).

The origin of the two electrophoretic variants of horse alpha-2 macroglobulin is unknown. Pepper (1968) has shown that the isolated macroglobulin is a dimer but that it occurs in whole serum as it's free subunit chains. However, Scott (1980, pers. comm.) has shown that the electrophoretic variants of horse alpha-2 macroglobulin revert to the mobility of the faster variant in the presence of excess trypsin, and similar observations have been reported in the case of human alpha-2 macroglobulin (Saunders et al., 1971; Ohlsson and Skude, 1976). Saunders et al., (1971) showed that the MW of the electrophoretic variants were identical and suggested that the macroglobulin may change it's shape and charge on interaction with the protease. This conflicts with the observations of Harpel (1973) who showed that the macroglobulin is broken into its subunit chains in the presence of proteases. This latter observation suggests that the heterogeneity may arise from fragmentation of the single polymeric protein during binding with endogenous proteases.

The two electrophoretic variants of horse alpha-2 macroglobulin at pH 7.35 possess a natural ability to hydrolyse ester substrates, in this case APNE, an observation also reported to be associated with human alpha-2 macroglobulin variants (Saunders et al., 1971). Whether this spontaneous esterase activity is associated with endogenous proteases bound to the macroglobulin is not established. Although Szewczuk and Szczeklik (1973) have shown that the amidase activity of the macroglobulin appears to be enhanced after complexing with thrombin during clotting, Saunders et al., (1971) have shown that the natural amidase activity of the five electrophoretic variants of human serum alpha-2 macroglobulin was identical and was not related to the degree of saturation with protease. In addition, these latter authors also showed that the natural amidase activity of serum alpha-2 macroglobulin was negligible in comparison to that of the fully saturated alpha-2 macroglobulin-trypsin complex, although the assay procedure, which employed a trypsin specific substrate[1], may have given a poor indication of the amidase activity of native alpha-2 macroglobulin.

The situation is further complicated by the presence of plasma pseudocholinesterases (Silver, 1974). These ubiquitous enzymes have been reported in the serum of many species (Augustinsson, 1961), and in the horse they possess an electrophoretic mobility similar to alpha-2 macroglobulin (Kaminski and Gajos, 1964). Furthermore these enzymes hydrolyse a wide range of ester substrates

1 Benzyol-DL-argenine-P-nitroanilide.

(Augustinsson, 1971), suggesting that the slow esterase zones (Es) in Figure 7.4 a correspond to pseudocholinesterase and that this pseudocholinesterase activity and the natural esterase activity of the alpha-2 macroglobulin may be the same entity. This hypothesis is supported by the similarity in pI of horse plasma pseudocholinesterase (pI 3.6-5.2) (Augustinsson, 1971) and horse alpha-2 macroglobulin (pI 4.8) (Lavergne and Raynaud (1970). Although the MW of the isolated dimeric macroglobulin from horse serum (1,000,000) (Pepper, 1968)) is greater than that of pseudocholinesterase (300,000-750,000) (Augustinsson, 1971, Lees and Harpst, 1973), the monomeric form of the macroglobulin occurring in fresh serum has a MW of 500,000 (Pepper, 1968). Borretti, Di Marco and Julita (1964) have shown that the sialic acid composition of horse alpha-2 macroglobulin is 3.6%, which is similar to that of the plasma pseudocholinesterase (3.2%) (Svensmark, 1965).

The inhibition of both horse and human pseudocholinesterase by di-isopropyl fluorophosphate shows that the enzyme is a serine hydrolyase (Kaminski and Gajos, 1964; Silver, 1974), the same as many endogenous proteases, both free (Dixon and Webb, 1979) and complexed with human alpha-2 macroglobulin (Barrett and Starkey, 1973). In human serum both the macroglobulin and this pseudocholinesterase possess an alpha-2 mobility on zone electrophoresis (Laurell, 1972[a]; Silver, 1974) and is eluted in the 19S peak on Sephadex G200 chromatography (Harris and Robson, 1963; Fireman, Vannier and Goodman, 1964). Four pseudocholinesterase isozymes and

four isoelectric variants of human alpha-2 macroglobulin have been described (Das and Liddell, 1970; Frenoy and Bourillon, 1974), although five esterolytically active electrophoretic variants of the macroglobulin were isolated by Saunders et al., (1971). Both the enzyme and the macroglobulin are glycoproteins whose pIs are similarly increased following neuraminidase desialation (Svensmark, 1965; Frenoy and Bourillon, 1974). The MW of human alpha-2 macroglobulin (725,000 ; Barrett and Starkey, 1973) is twice that reported for the pseudocholinesterase (350,000; Das and Lidell, 1970; Lockridge, Eckerson and LaDu, 1979) and Harpel (1973) has shown that the macroglobulin is made up of 4 subunit chains each of MW 185,000. Although Lockridge, et al., (1979) showed that human pseudocholinesterase is a tetramer made up of subunits of MW 90,000, Harpel (1973) has also shown that when α_2 macroglobulin interacts with endogenous proteases such as plasmin, thrombin and kallikrien the subunit chains are split into two fragments of MW 85,000. Razafirmahaleo and Bourillon (1968) have described an alpha-2 macroglobulin dissociation product whose 12S sedimentation coefficient compares with 12.4S recorded in the case of the pseudocholinesterase (Das and Liddell, 1970). Das and Liddell based their study on a pseudocholinesterase preparation isolated using, as a first step, DEAE chromatography (pH 4.0) and Harpel has shown that alpha-2 macroglobulin

will readily dissociate into subunit components at acid pH.

These observations indicate a remarkable similarity in the molecular properties of the plasma pseudocholinesterases and alpha-2 macroglobulin and suggest the two may be closely related, if not the same entity.

7.5. THE ELECTROPHORETIC HETEROGENEITY AND PROTEASE BINDING CHARACTERISTICS OF THE ALPHA-2 MACROGLOBULIN.

7.5.1. ELECTROPHORETIC HETEROGENEITY OF HORSE ALPHA-2 MACROGLOBULIN.

(i) Introduction

To investigate the occurrence of genetically determined polymorphism of alpha-2 macroglobulin, the electrophoretic heterogeneity of alpha-2 macroglobulin within a family based Thoroughbred population was examined. Starch gel electrophoresis was carried out at both pH 7.35 and pH 7.6. The latter method was based upon that used by Schroffel (1966) to demonstrate inherited polymorphism of pig alpha-2 macroglobulin.

(ii) Materials and Methods

(a) Sera: Sera obtained from the Thoroughbred family groups previously described in 6.6.1. was used. A number of sera from animals of unknown breeding were also used.

(b) Electrophoresis: Electrophoresis was carried out using the discontinuous phosphate-borate buffer system (pH 7.35) as previously described (7.3.2.). In addition a discontinuous tris/ cacodylic acid-borate buffer system (pH 7.6) was used as described by Kristjansson and Hickman (1965). This latter method was as follows:-

Gel Buffer: 2.29g Tris (hydroxymethyl) amino-methanine and 2.21g Cacodylic acid (dimethyl-arsonic acid) were made up to 1 litre in distilled water (pH 7.6).

Starch: 16% suspension of hydrolysed potato starch.

Tank Buffer: Tris-borate buffer (pH 8.6) was used as previously described (7.3.2).

Electrophoresis: Initially electrophoresis was carried out at 8.7 Vcm^{-1} (175V) for 20 minutes. Following removal of the inserts, electrophoresis was continued at 17.5 Vcm^{-1} (350V) until the borate boundary had migrated 7cms from the insert line. The gels were sliced and stained with 1% nigrosine (w/v) as previously described.

(iii) Results

In all sera examined by starch gel electrophoresis (pH 7.35) two distinct alpha-2 macroglobulin bands were apparent (Fig. 7.5a). In some sera a third, diffuse band of intermediate electrophoretic

Fig. 7.5. Starch gel electrophoresis of horse sera.

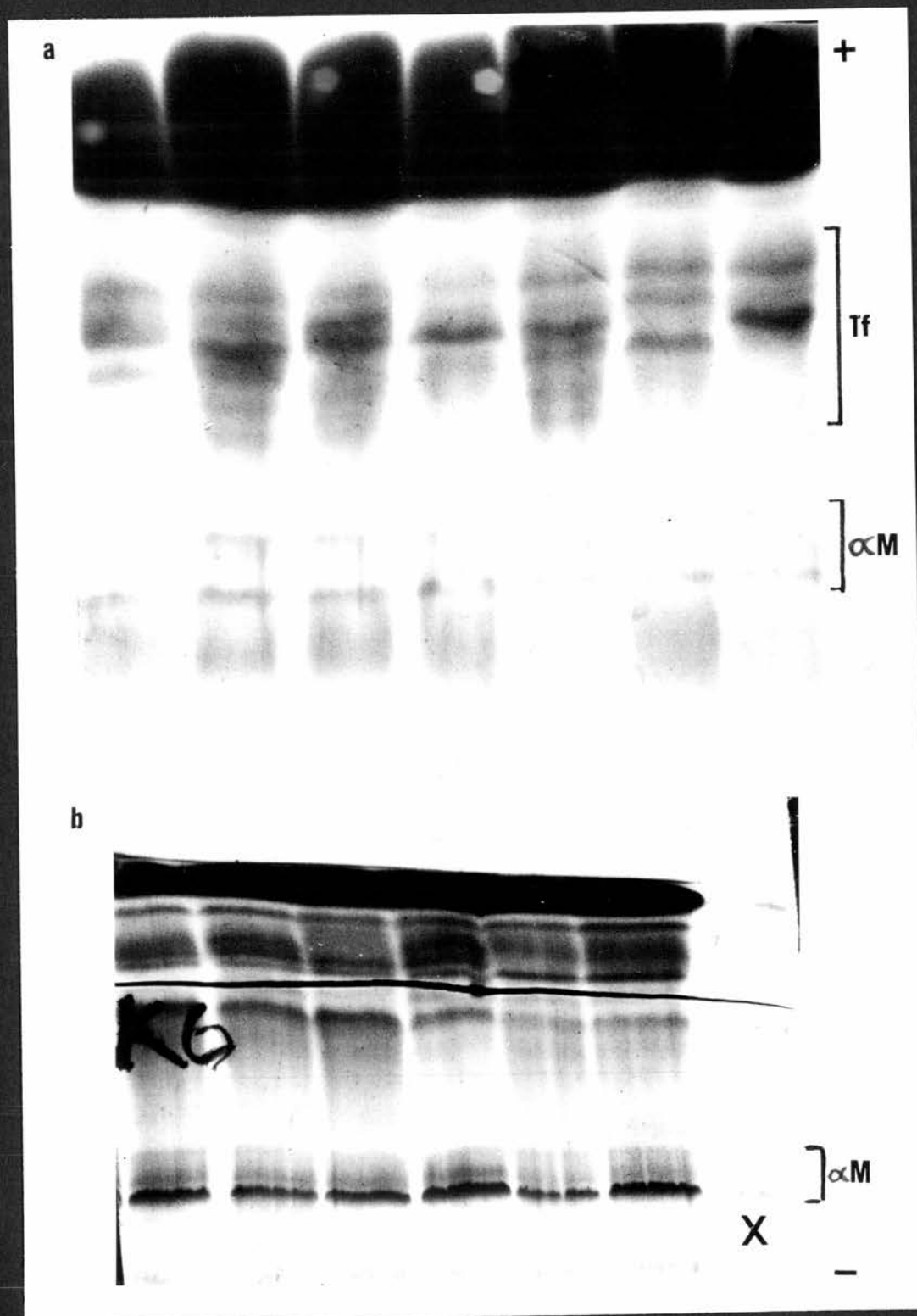
(a) pH 7.35

(b) pH 7.6, using a cacodylic acid-borate gel buffer.

X - Pooled Sephadex G200 fractions 10-13 (Fig. 7.1) of horse serum.

Tf - Transferrins.

αM - Alpha-2 macroglobulin.


a
+
Tf
αM
b
αM
X
–

mobility was apparent (Fig. 7.5a, sera 6 and 7), although whether this is a true macroglobulin variant is not known. Within the 34 Thoroughbred family groups no variation in the bimodal pattern of electrophoretic mobility of the alpha-2 macroglobulin was observed.

Following starch gel electrophoresis (pH 7.6) using a tris-cacodylic acid gel buffer alpha-2 macroglobulin, identified in pooled G200 fractions 10-13 (Fig. 7.5b), appeared as a single major band with two diffuse bands migrating immediately cathodal to the major variant. (Fig. 7.5b). Amongst the 34 Thoroughbred family groups, no variation in this pattern was observed.

Amongst the sera from animals of unknown breeding the alpha-2 macroglobulin band patterns apparent using both methods did not differ from those of the Thoroughbred.

(iv) Discussion

The alpha-2 macroglobin in horse serum is electrophoretically heterogeneous and at least three variants have been recognised. The molecular basis of these variants is unknown (7.4.3.). The albeit limited family data suggest that in the Thoroughbred inherited polymorphism of alpha-2 macroglobulins does not occur. The consistancy of the band patterns observed amongst the animals of mixed breeding suggests that inherited polymorphism of alpha-2 macroglobulin is unlikely to occur in horses as a species.

7.5.2. PROTEASE INHIBITORY ACTIVITY OF HORSE ALPHA-2 MACROGLOBULIN

(i) Introduction

To further examine the ability of the macroglobulin to inhibit the proteolytic though not the esterolytic activity of trypsin, the trypsin inhibitory capacity (TIC) of 8 whole sera and their pooled G200 fractions containing alpha-2 antiprotease activity were determined using both casein and benzoyl-DL-argenine-p-nitroanilide (DL-BAPNA) as trypsin substrates.

(ii) Materials and Methods

(a) Alpha-2 antiprotease: 8 fresh horse sera were subjected to Sephadex G200 chromatography as previously described (7.2.2.). The fractions containing alpha-2 antiprotease activity were pooled and concentrated by evaporation to the original serum volume 3.5ml and were dialysed against several changes of 0.01M PBS (pH 7.4) for 24 hours.

(b) Estimation of trypsin inhibitory capacity (TIC):

DL-BAPNA hydrolysis: The method used was based upon those described by Eriksson (1965) and Troyer and Moskowitz (1968) and is described in detail in Section 8.4. Briefly, whole serum or the serum fraction was incubated for 10 minutes at room temperature

with bovine trypsin in excess, after which time the substrate was added. The reaction was stopped after 20 minutes. Sample and trypsin controls were prepared by replacing the enzyme and serum respectively with buffer. The relative amounts of DL-BAPNA hydrolysed was expressed as the absorbance at 410nm, and the amount of trypsin inhibited by the serum (mg/ml) determined from a standard enzyme-substrate hydrolysis curve.

Casein hydrolysis: The experimental procedure was as described for DL-BAPNA hydrolysis except that 3% ($^{w}/v$) casein[1] in 0.2M Tris buffer (ph 7.6) was used as a substrate, and the reaction was stopped by adding 6ml of 2.5% ($^{v}/v$) trichloroacetic acid. The precipitated proteins were centrifuged out and the amount of split products in the supernatants determined by absorption at 280nm on an ultraviolet spectrophotometer[2].

(iii) Results

The TIC (mg/ml) of whole sera and of the pooled and concentrated Sephadex G200 fractions containing alpha-2 antiprotease activity determined using both casein and DL-BAPNA substrates are shown in Table 7.2.

1. Sigma Chemical Co., London.
2. Cecil Instruments, Cambridge, England.

TABLE 7.2.

TIC (mg/ml) OF WHOLE SERUM AND POOLED SEPHADEX G200 FRACTIONS CONTAINING ALPHA-2 ANTIPROTEASE ACTIVITY DETERMINED USING (a) DL-BAPNA (b) CASEIN SUBSTRATES.

SERUM	(a) DL-BAPNA SUBSTRATE		(b) CASEIN SUBSTRATE	
	WHOLE SERUM	G200 FRACTIONS	WHOLE SERUM	G200 FRACTIONS
1	1.20	0.10	1.60	0.65
2	1.90	0.20	1.60	0.85
3	1.40	0	1.55	1.20
4	1.40	0.05	2.10	0.90
5	0.60	0.20	1.60	0.75
6	1.50	0.05	2.20	0.70
7	1.00	0.10	1.60	0.70
8	1.25	0.10	1.80	0.85
$\bar{x} \pm$ 1 S.D.	1.28 ± 0.38	0.11 ± 0.08	1.76 ± 0.26	0.83 ± 0.16

With the exception of serum 2, the TIC of whole serum determined using a casein substrate was higher than that determined using the amide substrate. Statistical comparison of the mean TICs of whole horse serum determined using both methods show a significantly higher mean value using the casein substrate ($t = 2.9486$, d.f. 14; $0.005 < P < 0.01$).

The TIC of the alpha-2 antiprotease fractions, while constituting 48% of the total serum TIC when determined using the casein substrate, constitutes only 8.6% when measured using the amide substrate. These results indicate that, as in man, the alpha-2 macroglobulin-trypsin complex retains considerable activity towards amide substrates. Using the amide substrate method the absorbance at 410nm of both the whole serum and pooled G200 fraction sample controls was 0 in every case, indicating that the pseudocholinesterases and natural esterase activity associated with the alpha-2 macroglobulin are relatively inactive against DL-BAPNA and are unlikely to have influenced the amidase activity shown to be associated with the alpha-2 macroglobulin-trypsin complexes.

(iv) Discussion

The experimental data on substrate dependent variation in TIC of whole serum and alpha-2 macroglobulin support the earlier observations on the protease inhibitory activity of the macroglobulin (7.4), and

are similar to the observations of Troyer and Moskowitz (1968) on the protease inhibitory activity of human alpha-2 macroglobulin. These authors showed that the TIC of Sephadex G200 fractions containing alpha-2 macroglobulin, when determined using the amide substrate were less than one half those recorded using the casein substrate.

As in the report of Troyer and Moskowitz, the possible contribution of endogenous serum esterase components towards the amidase activity observed associated with the macroglobulin-trypsin complex in this study has been eliminated using sample controls. Nevertheless, in both this series and that of Troyer and Moskowitz the possible activation of amidase precursors in the test sera and serum fractions by trypsin cannot be discounted. However, DL-BAPNA is highly specific for trypsin (Dixon and Webb, 1979) and is unlikely to hydrolysed to any signficiant extent by endogenous esterases (Saunders et al., 1971).

The observations on protease binding by horse alpha-2 macroglobulin indicate that, as in man, in some way the macroglobulin is able to inhibit the proteolytic activity of the bound enzyme while still permitting access of small molecular weight substrates to its' catalytic site. Barrett and Starkey (1973) have suggested that this is due to steric inhibition resulting from a change in quaternary structure of the macroglobulin after binding to the enzyme, although

the results of both Harpel (1973) Topping and Seilman (1979) on the interaction of protease with the macroglobulin have challenged this hypothesis.

Alpha-2 macroglobulin accounts for 48% of the total antiprotease activity of horse serum. This figure compares with the 45% $\pm$ 2.7 figure reported by Von Fellenberg (1978b) for the percentage anti-trypsin activity of whole serum contributed by the alpha-2 antiprotease. This author also reported figures of 53.3% and 34% for the percentage anti-chymotrypsin and antielastase activity whole serum contributed by the alpha-2 antiprotease.

CHAPTER 8

SERUM Pr ANTIPROTEASE AND C.O.P.D. IN THE HORSE.

8.1. INTRODUCTION

The identity of the two major serum antiproteases in the horse has been established. Inherited polymorphism of only the electrophoretically faster, Pr, antiprotease can be demonstrated, and this protein appears to be analogous to human alpha-1 antitrypsin. To investigate the possible association of quantitative and genetic variation in the Pr antiprotease with the occurrence of C.O.P.D. in horses, analagous to alpha-1 antitrypsin dysproteinaemia in man, Pr allele frequencies and serum Pr concentration within a C.O.P.D. affected population were compared with those of healthy populations.

8.2. Pr ALLELE FREQUENCY WITHIN A C.O.P.D. AFFECTED POPULATION.

8.2.1. INTRODUCTION

To investigate whether horses of any particular Pr phenotype showed an increased predisposition to C.O.P.D., the Pr Allele frequencies within a C.O.P.D. affected population were determined and compared with those of healthy populations. All animals used in this study were affected with C.O.P.D. as defined by McPherson et al., (1978).

8.2.2. MATERIALS AND METHODS

(i) Sera

Serum was obtained from 64 horses and ponies affected with C.O.P.D., including 20 known Thoroughbred animals. The remainder were of mixed breeding. The sera had in some cases been stored for up to 7 years at -20°C.

(ii) Pr phenotyping

Pr phenotypes were determined using PGIEF (pH 4.0 - 6.0) as previously described (6.6.2.). The occurrence of the PrF allele product was confirmed using ASGE (pH 4.3) (6.6.1.).

(iii) Statistical Analysis

Differences in allele frequencies between populations were analysed using the G-test (Sokal and Rohlf, 1973) as applied by Buis (1976) to test the null hypothesis of absence of differences in the frequencies of serum protein alleles between pony populations. The G-test is a long likelihood ratio test similar to the chi-squared test and its test statistic, G, approximates numerically to X^2.

The null hypothesis of independence of Pr allele frequencies and the occurrence of C.O.P.D. was tested using the formula: $G = 2 [(\lessgtr f.\ln f$ cell frequencies) - ($\lessgtr f.\ln f$ row and column total frequencies) + $n.\ln n]$, where $n = \lessgtr f$.

8.2.3. RESULTS

The Pr phenotypes occurring within the C.O.P.D.

TABLE 8.1.

FREQUENCY OF Pr ALLELES IN HEALTHY PURE BRED HORSE AND PONY POPULATIONS AND IN MIXED BRED AND THOROUGHBRED C.O.P.D. AFFECTED POPULATIONS.

Breed[1] Author No. in Study		TB Scott (1976) 1500	Arab Scott (1976) 70	Shetland Pony Buis (1976) 280	Dole Pony Braend (1979) 400	WB Braend (1970) 111	Trotter Braend (1970) 111	TB[2] Present Study	C.O.P.D.[2] population 64 *	C.O.P.D.[2] population 20 **
A	F	0.057	0.170	0.014	0.102	-	0.032	0.073	0.106	0.125
L	G	0.030	0.097	-	-	0.010	-	-	-	-
L	I	0.077	-	0.046	0.004	0.060	0.045	0.040	0.055	0.075
E	L	0.447	0.351	0.395	0.070	0.210	0.207	0.556	0.297	0.300
L	N	0.177	0.009	0.070	0.635	0.090	0.203	0.032	0.062	0.125
E	S	0.079	0.135	0.345	0.156	0.090	0.374	0.145	0.148	0.225
S	T	-	-	-	0.008	-	0.063	-	-	-
	U	0.133	0.170	-	-	0.540	-	0.153	0.188	0.150
	W	-	-	0.130	0.025	-	0.077	-	0.148	-

1. TB = Thoroughbred; WB = Warmblood.
2. Determined by PGIEF (pH 4.0 - 6.0).

* Mixed bred
** Thoroughbred.

affected population are presented in Appendix 6. The frequency of occurrence of the Pr alleles within this population are presented in Table 8.1. along with the Pr allele frequency amongst the 20 Thoroughbred members of the population (Appendix 6). For comparison, the Pr allele frequencies of a number of horse breeds are also shown in Table 8.1.

The marked variation in Pr allele frequencies between different breeds restricts statistical comparison of healthy and C.O.P.D. affected populations to animals of a single type, in this case Thoroughbred. Between the healthy Thoroughbred populations shown in Table 8.1. (columns 1 and 7) the differences in Pr allele frequencies are significant ($G = 40.51$; d.f. $= 6$, $P < 0.005$), and therefore the frequencies within these two populations cannot be pooled for comparison with those of the C.O.P.D. affected Thoroughbred population (Buis, 1976). However, since the healthy Thoroughbred population used in the present study (Table 8.1., column 7) comprises of family groups derived from a limited number of sires (Table 6.5a) it cannot be considered unbiased (Buis, 1976), and comparison of healthy and C.O.P.D. affected Thoroughbred populations was made using the larger population of Scott (1977).

Differences in Pr allele frequencies between the C.O.P.D. affected Thoroughbred population and the healthy Thoroughbred population are significant at a

- 5% level ($G = 14.694$; d.f. = 6, $0.01 < P \leq 0.05$).

Within the overall C.O.P.D. affected population there was apparently an increased frequency of the electrophoretically slowest, PrW, allele. However, as discussed above, the significance of this observation within a mixed bred population cannot be statistically analysed.

8.2.4. DISCUSSION

Significant differences in allele frequencies were observed between healthy and C.O.P.D. affected Thoroughbred populations, probably associated with a relative increase in S allele frequency in the latter population. However, little clinical significance may be attached to this observation drawn from a small number of animals. In addition an increased frequency of the PrW allele was apparent within a genetically heterogeneous C.O.P.D. affected population. However, this observation does not vindicate the use of the W allele as a marker of the disease for a number of reasons. The PrW allele is found principally in pony breeds (Table 8.1) and McPherson *et al.*, (1979b) have shown that significantly greater numbers of ponies than horses are found within a C.O.P.D. affected population such as the one in this study, indicating that the increased PrW frequency may reflect the normal distribution of the allele within a healthy population of similar horse:pony numerical ratio.

Furthermore, the diagnosis of the PrW allele product after PGIEF (pH 4.0 - 6.0) may be confused by the overlap of the more anodal aliesterase bands into the Pr region (6.6.2). Error in identification of

the PrW product may also have arisen due to the age of the sera. Bacterial contamination and prolonged or indifferent storage may result in anomalous Pi patterns in human sera, in some cases associated with cathodal shifting of the bands (Lieberman et al., 1975; Ritchie and Smith, 1976; Inokuma et al., 1976), and prolonged storage of the C.O.P.D. sera used in the present study may have caused degeneration of the Pr or other, more cathodal, serum proteins producing a band whose pI is indistinguishable from that of the PrW product. In this respect, the occurrence of a presumed PrW allele product was most frequently recorded in sera which had been stored for the longest periods. To exclude misdiagnosis of the PrW product during PGIEF phenotyping, particularly after prolonged storage of sera, it would be necessary to demonstrate anti-protease activity associated with the suspected PrW bands using either a 'protease probe' or chromatic staining method.

From the results of this limited study, there is no evidence that any Pr allele acts as a marker for C.O.P.D.

8.3. SERUM Pr ANTIPROTEASE CONCENTRATION IN C.O.P.D. AFFECTED HORSES.

8.3.1. INTRODUCTION

Ek and Braend (1980) developed a radial immuno-

diffusion (RID) method of estimating the relative Pr concentration (Pr%) of horse serum, expressing the results as a percentage of a standard (100%) serum. Using this method they showed that amongst Norweigan Warmblood and Trotting horses homozygous PrS and PrU animals have significantly lower circulating Pr% levels than PrN and presumed Pr F, I, L and W homozygotes of the same age. Higher levels were found in foals irrespective of Pr phenotype, and no sex differences in Pr% levels were observed.

In the present study circulating Pr% levels within a C.O.P.D. affected population were determined using the RID technique and compared with those of the healthy populations studied by Ek and Braend (1980).

8.3.2. MATERIALS AND METHODS

(i) Sera

Thirty sera were selected from the C.O.P.D. affected population (8.2.1.). These were sera which had been stored at -20°C for the shortest period (< 2 years).

(ii) Serum Pr estimations

These estimations were carried out in collaboration with Nils Ek in the Department of Large Animal Medicine, Veterinary School of Norway using the modified Mancini RID method described in detail by Ek and Braend (1980). The Pr% levels are expressed as a percentage of a unit standard, Pr FF, serum.

8.3.3. RESULTS

The Pr% levels of the test sera are presented in Appendix 6. The mean $\pm$ 1 S.D. of the Pr% within the C.O.P.D. affected population is shown in Table 8.2 along with the mean $\pm$ 1 S.D. of the Pr% within 3 control populations of adult Warmblood and Trotter mares of Pr phenotypes NN, SS and UU (Ek and Braend, 1980). Ek (1979, pers. comm.) has quoted an approximate mean Pr% value of 82% within a healthy horse population of mixed or unknown Pr phenotype, and the range recorded within the Warmblood and Trotting horse population is 50-125% (Ek and Braend, 1980).

Statistical comparison of the mean Pr% levels of the C.O.P.D. population and the healthy PrNN population using Student's t test shows a highly significant difference ($t = 3.881$, d.f. = 51; $P < 0.001$). Genotypic variation in Pr% levels with the Pr phenotype may limit the validity of the t statistic derived from comparison of a mixed population with a purely PrN homozygote population. However, since the serum Pr% levels in the F, I, L and W homozygotes equate with those of the N homozygote (Ek and Braend, 1980) the increase in the Pr% levels in animals affected with C.O.P.D. is a true reflection of a quantitative response occurring during the course of the disease.

TABLE 8.2.

SERUM Pr% LEVELS (MEAN $\pm$ 1 S.D.) WITHIN A C.O.P.D. AFFECTED POPULATION AND THREE POPULATIONS OF ADULT WARM BLOOD AND TROTTING HORSES SEGREGATED ACCORDING TO Pr PHENOTYPE.

CLINICAL STATUS	n	Pr Phenotype[1]	Pr% $\bar{x}$ S.D.
Healthy	23	NN	83.7 ± 10.0[2]
Healthy	25	SS	76.4 ± 9.1[2]
Healthy	20	UU	70.7 ± 10.9[2]
C.O.P.D. Affected	30	Mixed	103.47 ± 26.46 Range (69-198%)

1. Limited data indicate that Pr% levels amongst F I L and W homozygotes are similar to those in PrNN individuals (Ek and Braend, 1980).
2. Ek and Braend (1980).

8.3.4. DISCUSSION

The RID results show significantly higher Pr antiprotease levels in the serum of C.O.P.D. affected animals than in the controls. The cause of these increased levels within the diseased population has not yet been established, although Ek and Braend (1980) noted increased Pr levels associated with a number of unspecified clinical conditions. Alpha-1 antitrypsin in human serum is an acute phase reactant protein and increases during inflammatory disease (Laurell, 1972b), and Talamo et al., (1972) showed that some human C.O.P.D. patients have raised serum alpha-1 antitrypsin levels. By analogy the elevated Pr antiprotease levels in the C.O.P.D. affected horses in this study may be the result of inflammation in the lower respiratory tract in these animals (1.1).

8.4. SERUM TRYPSIN INHIBITORY CAPACITY (STIC) IN NORMAL HORSES AND HORSES AFFECTED WITH C.O.P.D.

8.4.1. INTRODUCTION

Serum trypsin inhibitory capacity (STIC) is a commonly used and convenient measurement of anti-protease activity in whole serum or serum fractions (5.3.1). As the alpha-2 macroglobulin in horse serum strongly inhibits the proteolytic activity of trypsin but has only a limited effect on the amidase

activity of the enzyme, then STIC, measured using an amide substrate, may provide an estimate of Pr antiprotease activity. In man good correlation has been reported between STIC, determined using an amide substrate, and with immunochemical estimation of serum alpha-1 antitrypsin (Talamo et al., 1972; Dietz et al., 1974).

In this section the STICs of a healthy Thoroughbred population of varying sex and ages were compared with those of a C.O.P.D. affected population. In addition, the STICs and serum Pr% concentration amongst 30 individuals within the C.O.P.D. affected population were compared to determine the correlation and regression of Pr% on STIC.

8.4.2. MATERIALS AND METHODS

(i) Sera

The 30 C.O.P.D. affected animals used to determine circulating Pr% levels (8.3.2) were used, while 46 animals from a healthy Thoroughbred population (6.6.1), comprising of 20 mares, 14 foals and 12 stallions and geldings, were used as controls.

(ii) STIC determination

The method used was based upon those of Eriksson (1965) and Troyer and Moskowitz (1968) using the amide substrate benzoyl-DL-aginine-p-nitroanilide (BAPNA). P - nitro-aniline is released on tryptic hydrolysis of this substrate and may be measured

colourimetrically (Erlanger et al., 1961). STIC is an estimate of the ability of serum to inhibit this dydrolysis, and is expressed as mg trypsin inhibited per ml serum.

(a) Trypsin solution: Bovine pancreatic trypsin[1] was prepared in 0.0025M hydrochloric acid to a final concentration of approximately 225 µg/ml. The weakly acidic milieu prevents autolysis of the trypsin (Dietz et al., 1974). The exact trypsin concentration was determined as 0.585 absorbance at 280nm, measured using a Cecil CE292 Ultraviolet spectrophotometer[2]. To avoid variation in the assay procedure shown to result from variation in the purity of the enzyme (Dietz et al., 1967) a single batch of trypsin was used throughout. The trypsin solution was kept at 4°C for periods of up to 1 week.

(b) Substrate solution: A 0.08% (w/v) solution of BAPNA in distilled water was prepared by heating to 85°C. A 1:1.8 working dilution of substrate in 0.2M tris buffer (pH 7.6) was prepared immediately prior to each assay.

(c) Determination of trypsin activity: the enzyme concentration of increasing dilutions of the trypsin solution in 0.2M Tris buffer (pH 7.6) was calculated from the absorbance at 280nm, and 0.2ml aliquots of the dilutions were made up to 3ml with the working substrate solution and incubated at 37°C.

1 Sigma Chemical Co., London, England.

2 Cecil Instruments, Cambridge, England.

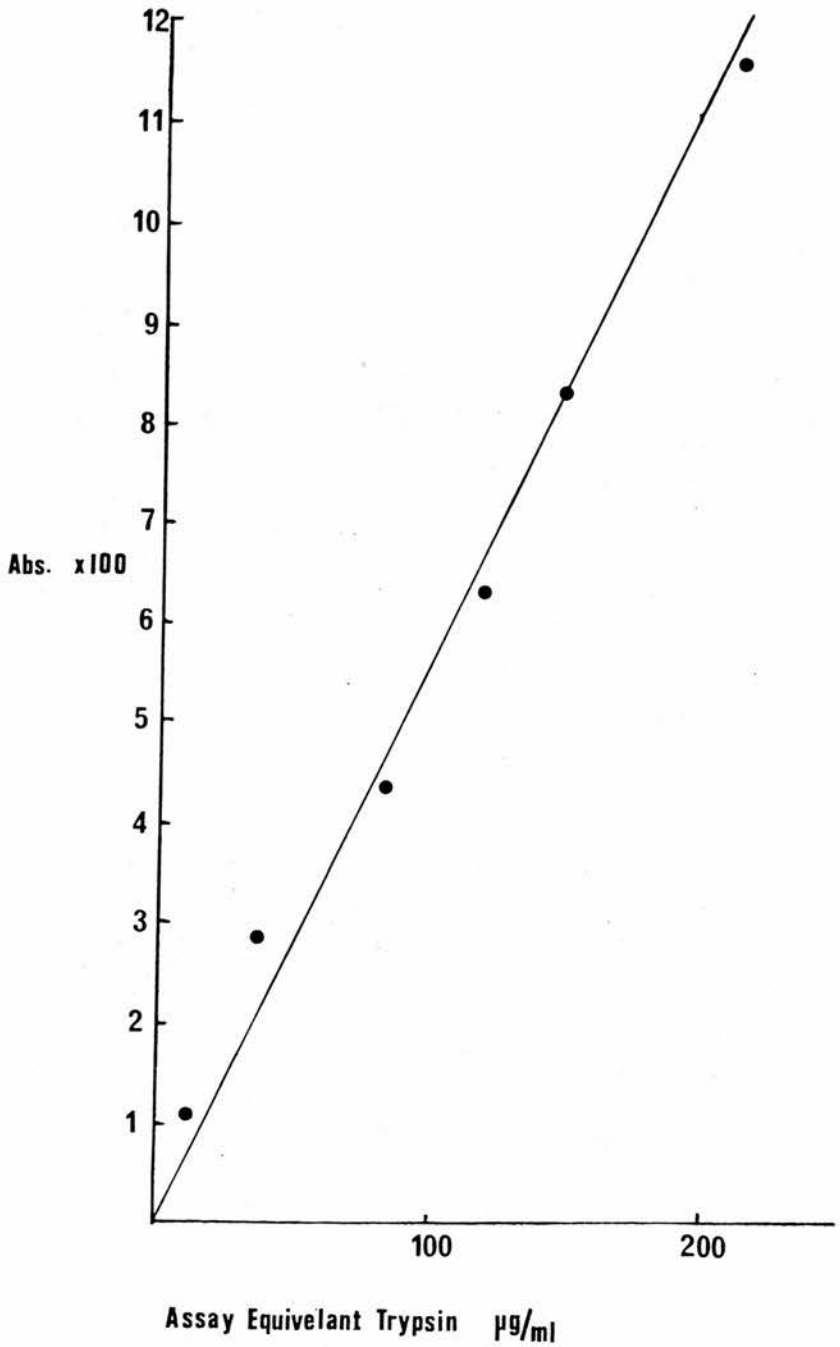


Fig. 8.1.

Hydrolysis of BAPNA by trypsin; sample absorbance at 410nm with increasing amounts of trypsin. Trypsin concentration is expressed as the assay equivalent, i.e. the trypsin concentration prior to dilution with the ester substrate solution.

The reaction was stopped after 20 minutes by adding 1ml 0.2M hydrochloric acid. Enzyme-free substrate blanks were prepared in the same way, and the absorbances of the test and control reactions were determined at 410 nm on the Cecil spectrophotometer. The absorbance of the substrate blanks in all cases were zero. The rate of tryptic hydrolysis of the substrate expressed as the absorbance at 410nm was plotted as a function of the assay equivalent trypsin concentration, i.e. trypsin concentration (µg/ml) prior to dilution with substrate, and is shown in Figure 8.1.

(d) Assay Proceedure: The test system is outlined in Table 8.3. All glassware was acid washed as recommended by Dietz et al., (1967).

The test serum was diluted 1:20 in 0.2M Tris buffer (pH 7.6) prior to assay so that the trypsin inhibitor concentration was less than the trypsin concentration. The working substrate solution was allowed to equilibrate at 37°C for at least 12 minutes before commencing stage 2.

After stopping the reaction at the end of Stage 2 the absorbance of the test and controls were determined at 410nm and in all cases the absorbance of the sample control was 0. The assay equivalent trypsin concentrations in the trypsin control and test solutions were estimated to the nearest 1.25 µg/ml from Figure 8.1 and the differences calculated. This

TABLE 8.3

OUTLINE OF PROCEDURE FOR ASSAYING TIC OF HORSE SERUM

(a) STAGE 1:

REAGENTS	TEST	Trypsin Control	Sample Control
0.2M Tris buffer (pH 7.6)	-	0.5ml	0.5ml
Trypsin solution	0.5ml	0.5ml	-
Diluted serum	0.5ml	-	0.5ml

Equilibrate mixtures at room temperature for 10 minutes.

(b) STAGE 2:

REAGENTS	TEST	Trypsin Control	Sample Control
Substrate/Buffer mixture	2.8ml	2.8ml	2.8ml
Trypsin/Sample mixture	0.2ml	-	-
Buffer/Sample mixture	-	-	0.2ml
Buffer/Trypsin mixture	-	0.2ml	-

Incubate at 37°C for 20 minutes before stopping reaction by adding 1ml 0.2M HCl.

difference was the amount of trypsin inhibited by 25µl serum. The figure was corrected to 1ml and was expressed as mg trypsin inhibited per ml serum.

Initially STICs were also calculated using the formula given by Eriksson (1965), though without the trypsin standardisation factor. This is;

$$\text{TIC (mg/ml)} = \frac{Ec - Es}{Ec} \times T \times \frac{1}{V}$$

where Es = absorbance of sample of 410nm

Ec = absorbance of trypsin control at 410nm.

T = amount of trypsin added (µg)

V = volume of serum added (µl)

(e) Statistical methods: The t-test for paired comparisons (Sokal and Rohlf, 1973) was used to test the differences in mean STIC of 8 sera derived using the graphical method and the mathematical method respectively.

The experimental error of the assay procedure was estimated from duplicate determinations on 8 sera and was expressed as the standard error of a single determination where $SE = \sqrt{\frac{\leqslant i^2}{2N}}$, i being the differences between the duplicate results and N the number of double determinations (Dahlberg, 1940). The reproducibility of the method was calculated as the coefficient of variation of 8 repeat determinations on a single serum, carried out on different days (Talamo, et al., 1972).

The mean STIC of subgroups of the healthy population segregated according to age and sex were compared using Student's 't' test. The mean STIC of the healthy population and the C.O.P.D. affected population were also compared using Student's 't' test.

The relationship of serum Pr concentration (Pr%) and STIC within the C.O.P.D. affected population was investigated by regression analysis (Smart, 1970). The correlation coefficient of STIC and Pr% was calculated, and the significance of both the regression and correlation coefficients was determined according to the 't' distribution (Smart, 1970).

8.4.3. RESULTS

(i) Experimental Error

The STICs of 8 sera calculated using the both graphical method and the mathematical method based on Eriksson's formula are presented in Appendix 4a. No significant differences between the paired readings were demonstrated using the t-test for paired comparisons (t_s = 0.0874; d.f. = 7, $P > 0.05$) and subsequent determinations were based solely on the graphical method, obviating the need for determining the working trypsin concentration prior to each assay.

The SE of a single STIC estimation determined from duplicate estimations on 8 sera (Appendix 4b) is 0.157 mg/ml, which corresponds to 11.1% of the mean STIC of the healthy population (Table 8.3).

This S.E. is greater than the 0.02mg/ml error (2% of the mean STIC) quoted by Eriksson (1965) for his own method, which corresponds to an error in the absorbance of approximately 0.003 in contrast to approximately 0.025 in the present study.

The mean ± 1 S.D. of the differences between the 8 duplicate STIC estimations in the present study is 0.05 ± 0.231mg/ml, which is similar to the mean difference in duplicate STIC measurements reported by Dietz et al., (1974) (0.03 ± 0.23 µmol/min./ml) where the population mean STIC is around 3.0 µmol/min./ml.

The coefficient of variation (CV) of 8 repeat STIC estimations on a single serum (Appendix 4c) is 10.3% (mean ± 1 S.D. = 1.68 ± 0.173; Range 1.45 - 1.95). Eriksson (1965) reported CVs of 4.6% and 3.0% for repeat STIC determinations on 2 sera of TIC range 0.81 - 0.92 and 1.28 - 1.38 respectively although Talamo et al.(1972) later recorded CVs of 5.6% and 46.5% after repeat assays on normal and homozygous alpha-1 antitrypsin deficient serum respectively.

(ii) STIC of normal and C.O.P.D. affected populations

Individual STICs (mg/ml) of the healthy and C.O.P.D. affected populations are presented in Appendices 5 and 6 respectively. Statistical analysis of the differences between the mean STIC of

the healthy foals and adults, and the healthy mares and geldings are shown in Table 8.4 a and b respectively. Statistical analysis of the differences between the mean STIC of the healthy and C.O.P.D. affected populations is shown in Table 8.4,c. No significant difference was observed ($P > 0.05$) in any of these 3 comparisons.

(iii) Association of STIC with Pr phenotype

The Pr phenotypes of the individuals within the healthy population are presented in Appendix 5. The distribution of STIC in relation to Pr phenotypes in this population (Fig. 8.2.) shows no apparent association of Pr phenotype with STIC. Although the Pr SS, SU and UU STIC lie below the mean, greater numbers of these phenotypes are necessary to establish their association with low STIC values comparable to their association with low Pr% (Ek and Braend, 1980).

(iv) Association of STIC and Pr%

The correlation and regression coefficients of STIC and Pr% of 30 animals within the C.O.P.D. affected population (Appendix 6) are shown in Table 8.5 along with their significance calculated according to the 't' distribution.

TABLE 8.4

STATISTICAL COMPARISON OF THE MEAN STIC (mg/ml) OF AGE AND SEX SEGGREGATED SUBGROUPS OF A HEALTHY THOROUGH-BRED POPULATION, AND OF THIS HEALTHY POPULATION WITH C.O.P.D. AFFECTED POPULATION.

(a)

GROUP	$\bar{x} \pm 1$ S.D. (Range)	t d.f.=44	Significance P
T.B. Adults (>1 year old) (n=32)	1.43 ± 0.44 (0.65 - 2.25)	0.5295	n.s.*
T.B. Foals (n=14)	1.36 ± 0.40 (0.75 - 2.20)		

(b)

GROUP	$\bar{x} \pm 1$ S.D. (Range)	t d.f.=30	Significance
T.B. Mares (n=20)	1.48 ± 0.45 (0.65 - 2.20)	0.8368	n.s.*
T.B. Stallions and geldings (n=12)	1.35 ± 0.41 (0.80 - 2.25)		

(c)

POPULATION	$\bar{x} \pm 1$ S.D. (Range)	t d.f.=74	Significance
Healthy T.B. Population (n=46)	1.41 ± 0.42 (0.65 - 2.25)	0.4725	n.s.*
C.O.P.D. Affected population (n=30)	1.36 ± 0.47 (0.75 - 2.45)		

* n.s. - not significant, $P > 0.05$.
d.f. - degrees of freedom.

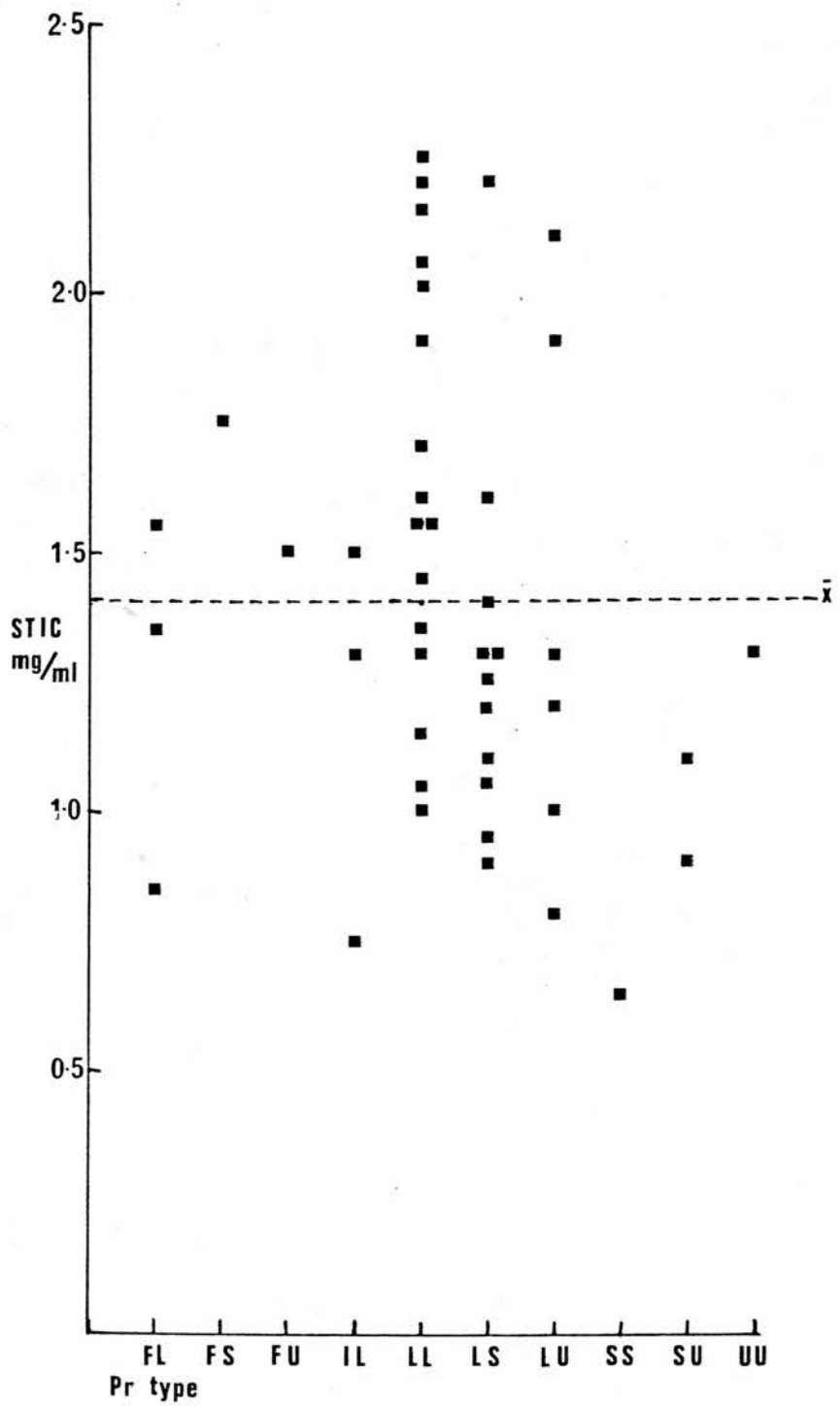


Fig. 3.2. Distribution of STIC in relation to Pr phenotypes observed within a healthy Thoroughbred population, $\bar{x}$ denotes the population mean STIC.

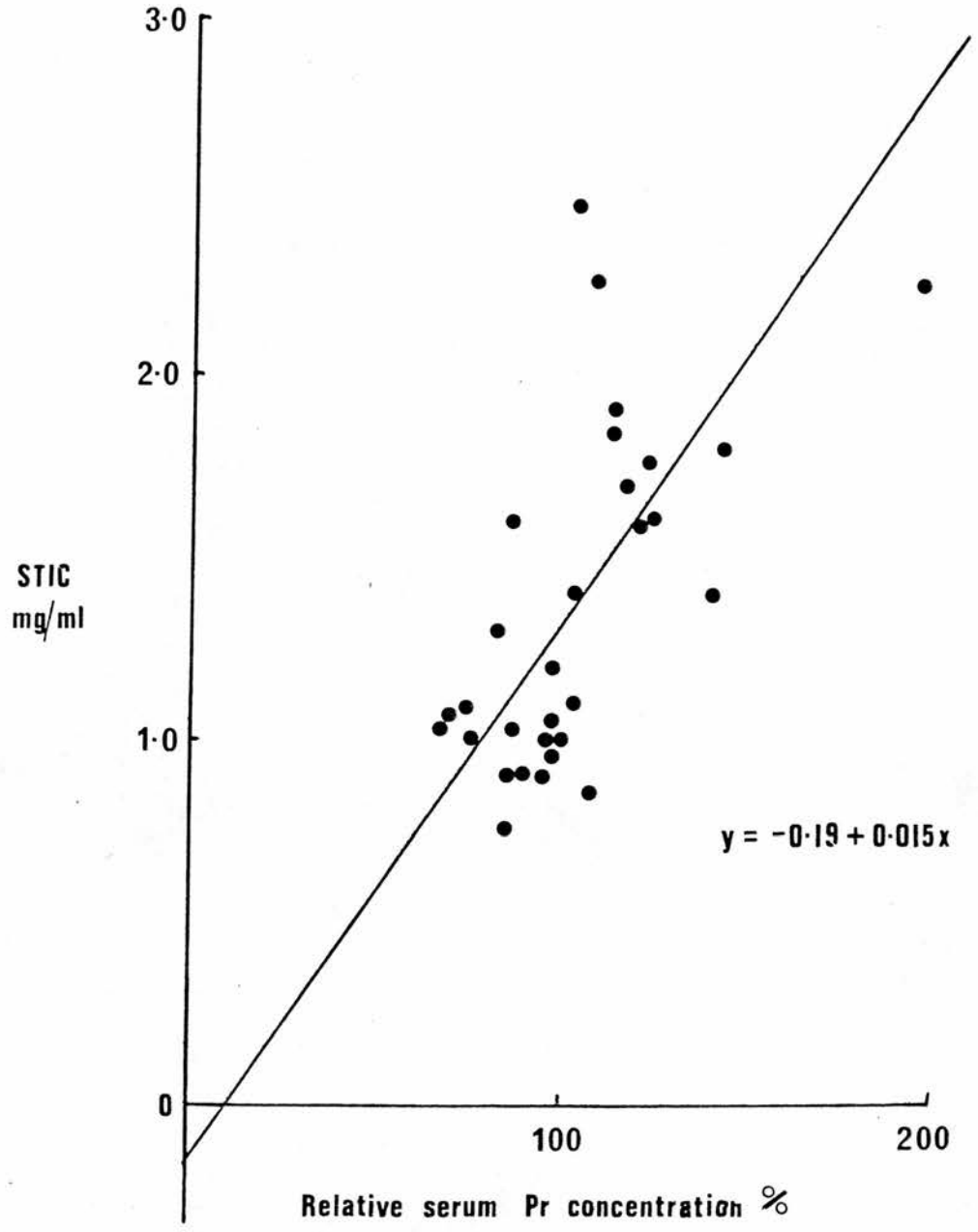


Fig. 8.3. Scatter diagram showing the relationship of STIC and Pr% amongst a group of 30 C.O.P.D. affected animals.

The regression of STIC on Pr% is expressed by the equation y = -0.19 + 0.015X.

TABLE 8.5.

STATISTICAL SIGNIFICANCE OF THE CORRELATION AND REGRESSION COEFFICIENTS OF THE STICs AND RELATIVE SERUM Pr CONCENTRATION WITHIN THE C.O.P.D. AFFECTED POPULATION (n = 30).

		t	P
Coefficient of Correlation:	0.827	13.848	$P < 0.001$
Coefficient of Regression:	0.015	7.895	$P < 0.001$

Both the regression and correlation coefficients are highly significant ($P < 0.001$).

STIC plotted against Pr% is shown in Figure 8.3 and the linear regression of STIC on Pr% is expressed by the equation $Y = -0.19 + 0.015X$.

8.4.3. DISCUSSION

The STIC assay procedure described in this study differs from that described by other authors in a number of minor ways. Firstly, Dietz et al., (1974) included albumin in the trypsin control to eliminate potential error arising due to the non-linearity of the trypsin activity-serum concentration relationship at low serum concentrations ($< 1\mu l$) (Dietz et al., 1967). However at the serum concentration used in the present study ($5\mu l$) this relationship is linear (Dietz et al., 1967) and the inclusion of albumin in the trypsin control was considered unnecessary. Secondly, Eriksson (1965) found that with increasing

volumes of serum (> 40µl) the relationship of serum concentration and percentage trypsin inhibition became non-linear when the percentage inhibition exceeded 80%. To overcome this, the author routinely estimated the TIC of two independent dilutions of each serum to ensure the TIC fell within the linear part of the serum concentration-percentage inhibition curve. However the 5µl serum volumes used in the present study falls well within the linear part of this curve and in no instance did the inhibition exceed 80% of the trypsin in the assay. Thirdly, Eriksson (1965) standardized the commercial trypsin preparations used in the assay using soya bean trypsin inhibitor (STI) and corrected the TIC accordingly. However, as stated by Dietz et al., (1967) this standardization is dependant upon the purity of the STI and the accuracy of the molecular weights and combining ratios assigned to STI and trypsin. Furthermore the standardization does not affect the accuracy of the results although it does cause difficulty in comparison of the results from different laboratories.

Amongst the C.O.P.D. affected group a significant linear relationship was observed between the STIC levels measured using the BAPNA substrate and the serum Pr% levels within the limits of the data. Similar observations have been recorded in relation to alpha-1 antitrypsin levels in man (Talamo et al., 1972;

Deitz, et al., 1974) despite the limited inhibitory activity of human alpha-2 macroglobulin on trypsin esterolysis (Ganrot, 1966b). However, since data already presented in Table 7.2 shows that in the horse alpha-2 macroglobulin contributes some 9% of the total STIC when measured using an amide substrate, the regression line of STIC against Pr% would be expected to transect the Y axis at a positive intercept rather than the -0.19 value established (Fig. 8.2). This negative intercept on the Y axis may indicate that some of the Pr protein measured by RID may possess no antiprotease activity.

The mean STIC of 46 normal horses in the present series (1.41 $\pm$ 0.42 mg/ml) is significantly lower than that reported for a control horse population by Breeze et al., (1977) (1.60 $\pm$ 0.31; $n = 28$, $t = 2.2288$; $0.01 < P < 0.05$). Although the technique used in both instances was based upon those of Eriksson (1965) and Troyer and Moskowitz (1968), differences may have arisen from variation in purity of the trypsin used in the respective assays. Furthermore the animals used by Breeze et al., were not described as clinically healthy but simply as free from clinical signs of respiratory disease and many may have been hospitalised animals. Disease associated increases in serum Pr levels have been observed (Ek and Braend, 1980), and may account for

the raised STIC in such a control population.

In the present series, no difference was apparent between the mean STIC of the mares and geldings/stallions in the healthy population, and no difference was recorded between the mean STIC of the foals and adults within this population. Ek and Braend (1980) however have recorded significant differences in the relative Pr levels of foals and adults in a population of Scandinavian native horses, however quantitative electrophoresis of foal serum (Morgan, 1972) has indicated lower alpha-2 macroglobulin levels in foals compared to adults, which may account for the similar STICs found in the foal and adult subgroups, despite a possible increase in Pr levels in the former group.

A similar discrepancy was observed between the mean STIC and %Pr levels in the C.O.P.D. affected animals in comparison to the healthy population. The similar STIC levels in contrast to significantly raised relative Pr% levels in the C.O.P.D. population may be due to the increased Pr levels, as part of the inflammatory response, consisting of relatively higher levels of a non functional component. Such a situation could arise should the non functional Pr component represent circulating Pr-leucoprotease complexes.

In conclusion, this study of antiprotease deficiency and C.O.P.D. in the horse has failed to demonstrate the occurrence of antiprotease deficiency in the horse analogous to alpha-1 antitrypsin in man, and has further failed to demonstrate any association of lowered circulating antiprotease levels with the onset of C.O.P.D.

SERUM ANTIPROTEASES AND C.O.P.D. IN THE HORSE: GENERAL DISCUSSION AND CONCLUSIONS.

The major antiproteases in horse serum have been identified and characterised, and a number of differences are apparent between the serum antiproteolytic mechanisms in the horse and man.

Firstly, alpha-2 macroglobulin accounts for a greater proportion of serum antiprotease activity in the horse than in man. Secondly, there is no evidence that any allele of the Pr system, the horse homologue of human alpha-1 antitrypsin, results in significantly lower circulatory levels of the antiprotease comparable to the PiS and PiZ alleles in man. Furthermore, the distribution of Pr alleles amongst horse and pony populations is wide in comparison to the distribution of Pi alleles in man, suggesting that no phenotype is biologically disadvantageous. An exception to this is the PrG allele, whose frequency in the Thoroughbred is very low and whose most anodal product possesses no antiprotease activity (Scott, 1980; pers. comm.). However the resulting deficiency in overall serum antiprotease activity in even the PrG homozygous animal is likely to be so marginal as to be clinically insignificant. Thus, an inherited deficiency in serum antiprotease activity, analgous to alpha-1 antitrypsin deficiency in man, probably does not occur in the horse, and may be discounted as a significant predisposing factor to the onset of C.O.P.D.

However, since equine C.O.P.D. involves hypersensitization to inhaled microbial antigens, these

observations on serum antiproteolysis do not exclude local antiprotease deficiency in the bronchiolar lumen from the pathogenesis of the disease. Since the horse lacks a lung specific inhibitor then serum derived antiproteases may be important in respiratory surface defence against leucoproteases and microbial proteases which may occur in the lower airway during C.O.P.D. Should the transfer of antiproteases from the circulation to the bronchiolar lumen be compromised, as for instance by active bronchiolar inflammation, then the respiratory tract may remain unprotected against further proteolysis. However much more detailed investigation of the antiprotease defence mechanisms in horse lung are necessary before such a hypothesis can be substantiated.

SECTION 3

SOME PRELIMINARY OBSERVATIONS ON THE NATURE OF AN EQUINE HOMOCYTOTROPIC OR REAGINIC ANTIBODY.

CHAPTER 9

REVIEW OF THE LITERATURE

9.1. IMMUNE MECHANISMS OF TISSUE DAMAGE

Von Pirquet (1906) first used the term allergy to describe the altered reactivity of a host to a foreign substance or allergen. This was subsequently modified by Gel and Coombs (1963) who proposed that 'allergy' be used to describe the specifically altered state of a host following exposure to the allergen. Although Von Pirquet (1906) realised that a number of fundamentally different allergic processes may occur simultaneously, Gel and Coombs (1963) were the first to define different types of allergic reaction or hypersensitivity, proposing four mechanisms of immunologically mediated tissue damage:

Type I - Reaginic or homocytotropic antibody dependant hypersensitivity; the anaphylactic reaction.

Type II - Antigen-antibody cytotoxic or cell surface membrane reactive hypersensitivity.

Type III - Antigen-antibody complex mediated hypersensitivity; the Arthus reaction.

Type IV - Delayed or cell mediated hypersensitivity.

Recently, the 'cell mediated' reactions have been subdivided as follows according to the effector cell type involved (Denman, 1977).

(i) T (thymus) lymphocyte mechanism; target cell killing independent of antibody.

(ii) K (killer)-cell mechanism; destruction of antibody coated target cells by Fc receptor bearing mononuclear cells.

(iii) Macrophage mechanism; macrophages may become cytotoxic in the presence of both antigen - IgG complexes and a specific antibody-like macrophage arming factor.

9.2. HOMOCYTOTROPIC ANTIBODIES IN MAN

9.2.1. HISTORICAL

Prauznitz and Küstner (1923), and later De Besche (1923) and Freeman (1924) demonstrated a serum factor in allergic individuals capable of passively sensitizing homologous skin to the specific allergen involved. These serum factors were termed 'atopic reagins' by Coca and Grove (1925) who first demonstrated their characteristic heat lability and their prolonged persistance in homologous skin. These reagins neither fixed complement, nor acted as precipitating or neutralising antibodies (Coca and Grove, 1925), and were salt precipitated in the pseudoglobulin fraction (Scherer, 1931; Stull, Sherman and Cooke, 1938). Bell and Eriksson (1931) demonstrated the inability of reagins to cross the human placenta.

The earliest method of detecting serum reagins was the Prauznitz-Kustner (P-K) test which involved intradermal (I/D) inoculation of allergic serum followed, after an appropriate sensitization interval, by I/D

antigen challenge at the same site resulting in an immediate localised wheal and flare reaction.

Early studies on the physicochemical properties of reagins have been reviewed in detail by Stanworth (1963). However, attempts to isolate reaginic antibodies using ion-exchange chromatography techniques were unsuccessful, although a characteristic pattern of elution of serum P-K activity was established (Humphrey and Porter, 1957; Stanworth, 1959).

Following the identification of human immunoglobulin A preliminary studies suggested that reaginic activity may be associated with this immunoglobulin class (Heremans and Vaerman, 1962; Fireman, Vannier and Goodman, 1963). However, Loveless (1964) demonstrated P-K activity in serum with no detectable IgA, and Stanworth (1965) showed that the elution of IgA on ion exchange chromatography did not exactly parallel that of serum P-K activity. Subsequent investigations showed that the reagins did not belong to any of the four established immunoglobulin classes; IgG, IgM, IgA or IgD (Ishizaka et al., 1965; Ishizaka and Ishizaka, 1966; Ishizaka, Ishizaka and Lee, 1966) but that they probably belonged to a new immunoglobulin class tentatively called immunoglobulin E (IgE) after their specific binding affinity for ragweed allergen E (Ishizaka, Ishizaka and Hornbrook, 1966a). Using radiolabelled ragweed

allergen E these authors were able to correlate the reaginic activity of sera from ragweed hypersensitive individuals with serum IgE levels (Ishizaka, Ishizaka and Hornbrook, 1966b; 1967). Reaginic antibody was finally isolated using a complex multistep fractionation procedure and was definitively identified as the unique immunoglobulin class IgE (Ishizaka and Ishizaka, 1967).

A myeloma paraprotein (IgND) which was antigenically unrelated to any of the known immunoglobulin classes was described by Johansson and Bennich (1967) and Johansson, Bennich and Wide (1968), and was shown to occur in low levels in the serum of normal individuals (Johansson, 1968). Subsequently, anti-Fc IgND was shown to result in a single precipitin arc against isolated human IgE (Bennich, et al., 1969), and Stanworth, et al., (1967) demonstrated competitive inhibition of reagin mediated passive skin sensitization by both IgND and its Fc fragment. Bennich et al., (1968) prepared a memorandum for the World Health Organisation proposing that reaginic antibodies be considered representative of a new immunoglobulin class, with the heavy polypeptide chains referred to as epsilon (ε) chains. However, IgE producing myelomas in man are rare and between 1967 and 1976 only nine cases were reported in the literature (Bennich, Johansson and Bahr-Lindstrom, 1976).

9.2.2. HOMOCYTOTROPIC ANTIBODY FUNCTION

Increased serum concentrations of IgE are characteristic of atopic disorders in man. Atopy has been defined as a spontaneous tendency for an individual to produce high levels of IgE reacting with one or more antigens, in association with antigen-provoked disorders, in some or all of which a reaginic mechanism can be identified (Reeves, 1977). Typical atopic disorders associated with elevated IgE levels are extrinsic asthma, hay fever and atopic dermatitis (Johansson, Bennich and Berg, 1972). Raised serum IgE levels also occur in association with parasitic infestation, particularly ascariasis and visceral larva migrans (Johansson, Bennich and Berg, 1972). Kay (cited by Watts, 1979) has hypothesised that atopy, virtually absent from parasitised populations, represents an aberrance of an IgE mediated anti-parasitic immune response in parasite free populations.

9.2.3. IMMUNOCHEMICAL PROPERTIES OF HUMAN IgE

The extensive literature on the physiochemical and immunological properties of human IgE has been reviewed in detail (Bennich and Johansson, 1971; Ishizaka and Ishizaka, 1975; Bennich *et al.*, 1976; Bennich, Johannsson and Bahr-Lindstrom, 1976), and the principal molecular properties may be summarised as follows:

(i) 8.2S glycoprotein, M.W. 190,000.

(ii) Gamma-1/Beta electrophoretic mobility at pH 8.6.

(iii) The epsilon polypeptide chain comprises four constant domains (CE1, CE2, CE3, CE4) and one variable domain (VE). Two groups of isotypic antigenic determinants, DE1 and DE2, are found in the CE2 and CE3-CE4 domains respectively. The cell fixing site(s) are probably located on the carboxy terminal region of the epsilon chain, probably in the CE3-CE4 domains.

(iv) IgE is heat labile at 56°C and 2-4 hours, and sensitive to the thiol reducing agents, 2-mercaptoethanol and dithiothreitol.

(V) IgE does not activate complement via the classical pathway, although there is some evidence of activation via the alternative pathway.

(vi) IgE sensitizes homologous skin (including monkey skin) with an optimum sensitization time prior to antigen challenge of 1-3 days. The I/D half life of IgE is approximately 13 days.

9.2.4. CYTOTROPIC ACTIVITY OF IgE

IgE has been shown to bind to specific receptor sites on the plasma membranes of both tissue mast cells (Tomoika and Ishizaka, 1971) and circulating basophil granulocytes (Sullivan, Grimley and Metzger, 1971). Bennich and Bahr-Lindstrom (1974) have hypothesised that the cell membrane binding mechanism involves two distinct sites on the CE3-CE4 domain of the IgE heavy chain. A primary site recognises the basophil or mast

cell membrane receptor, and a secondary site, with no particular cell specificity, then interacts with the membrane receptor after antigen binding.

The exposure of sensitized basophils to allergen (direct anaphylaxis) (Levy and Osler, 1966) or anti-IgE (reversed anaphylaxis) (Ishizaka and Ishizaka, 1969) results in the release of a heterogeneous group of chemical mediators of the allergic reaction; principally histamine, 5 H-T, bradykinin, slow releasing substance-A (SRS-A), eosinophil chemotactic factor (ECF) and prostaglandins (Valentine 1976). The sequence of complex intracellular biochemical and physiological events occurring during release of these pharmacologically active compounds have been recently reviewed by Kasimierczak and Diamant (1978).

The immunological mechanism initiating degranulation appears to involve the crosslinkage of two cell bound IgE molecules, either by bridging of their $F(ab^1)_2$ moeities by specific divalent antigen or hapten (Siraganian, Hook and Levine, 1975) or by bridging their Fc antigenic determinants by the $F(ab^1)_2$ moeity of a divalent anti-IgE molecule. (Ishizaka and Ishizaka, 1969). This 'bridging' hypothesis of immune mediated basophil degranulation has been discussed by Stanworth (1973) and Ishizaka and Ishizaka (1975) and recently, Ishizaka, et al., (1978) have demonstrated histamine release from non-sensitized rat leukaemic basophils by the $F(ab^1)_2$ fragment of IgG antibody raised

against the IgE membrane receptor molecules on these cells, suggesting that the cross linkage of the membrane receptor sites rather than the IgE molecules is the primary factor in the activation of the degranulation process.

9.2.5. CLASS HETEROGENEITY OF HOMOCYTOTROPIC ANTIBODIES IN MAN

On DEAE anion exchange chromatography of allergic serum, P-K activity is eluted over a wide range of increasing molarity (Humphrey and Porter, 1957; Fireman, Boesman and Gitlin 1967; Reid, Minden and Farr, 1968), although whether this distribution represented the association of reaginic activity with more than one immunoglobulin class was not established. Subsequently Radermecker (1969) and Reid (1970) reported reaginic activity in 'pure' IgG preparations, although, unlike IgG antibodies, this activity was sensitive to heat and thiol reducing agents and possessed an I/D half life of 8 to 12 days.

However, Parish (1970; 1974) showed that IgG fractions of allergic sera with no detectable IgE could passively confer anaphylactic sensitivity on monkey skin but not on guinea pig skin. This 'non-reaginic' homocytotropic antibody was heat stable and thiol reducing agent resistant, and sensitized homologous skin only up to 24 hours. The skin sensitizing activity appeared complement dependent (Parish 1970) and could be precipitated out of serum by anti-IgG, though not by anti-IgE (Parish 1974; Bryant, Burns and

Lazarus, 1975). In addition, high levels of this short term skin sensitizing antibody (IgG-ST) were reported in the serum of asthmatic individuals with normal levels of IgE and who were non-responsive to disodium chromoglycate, an effective inhibitor of IgE mediated hypersensitivity. (Bryant, Burns and Lazarus, 1973).

Although Stanworth and Smith (1973) demonstrated competitive inhibition of the skin sensitizing activity of IgE by human IgG_4 myeloma paraprotein, Parish (1974) first proposed an association of IgG-ST with human IgG_4. This hypothesis was strengthened by the results of Vijay and Perelmutter (1975) showing histamine release from sensitized leucocytes following exposure to anti-IgG_4 serum.

9.3. CYTOTROPIC ANTIBODIES IN THE HORSE

9.3.1. HISTORICAL

Ritzenthaler (1924) demonstrated the occurrence of homocytotropic-like antibodies in the horse by the passive transfer of systemic anaphylactic sensitivity to a normal horse by intravenous (I/V) administration

of serum from a horse experimentally sensitized to ovalbumin. However this demonstration of homologous passive transfer of systemic anaphylactic sensitivity in the horse has not been repeated.

9.3.2. HETEROCYTOTROPIC ANTIBODIES IN THE HORSE

Avery and Tillet (1929) and Mehlman and Seegal (1934) demonstrated systemic anaphylaxis in guinea pigs after intraperitoneal (I/P) sensitization with antipneumococcal rabbit serum followed 24-72 hours later by I/V pneumococcal antigen challenge, but were unable to repeat these results using anti-pneumococcal horse serum. The former authors attributed this discrepancy to 'anti-complement' activity in horse antipneumococcal sera (Zinsser and Parker, 1923), although Mehlman and Seegal (1934) were unable to demonstrate differences in circulating complement levels following sensitization of guinea pigs with horse antipneumococcal serum. Mehlman and Seegal (1934) however, demonstrated passive sensitization of guinea pig skin by I/P inoculation of antipneumococcal horse serum followed 24 hours later by I/D antigen challenge. The localised anaphylactic nature of the dermal reaction was indicated by the rapid extravasation of circulating dye at the antigen inoculation sites.

The inability of I/V administered antipneumococcal horse serum to confer systemic anaphylactic sensitivity on guinea pigs was subsequently confirmed by

Benaceraff and Kabat (1950), who showed however that I/D antigen challenge 30 minutes after I/V sensitization resulted in a local Arthus-like haemorraghic reaction, apparent within 30 minutes and reaching a maximum after two hours. These findings were later confirmed by Ovary and Bier (1953) who also described and established the Passive Cutaneous Anaphalaxis (PCA) test as a method of detecting cytotropic antibodies. This test involves initial I/D sensitization of homologous or heterologous skin with test serum followed after an appropriate sensitization time by I/V injection of both specific antigen and marker dye. A positive reaction, indicative of homo- or heterocytotropic antibodies in the test sera, is indicated by localised colouring of the dermis at the sensitized sites within 10 to 15 minutes of antigen challenge. Using this technique in guinea pigs sensitized I/D for 3-6 hours with hyperimmune antityphoid horse serum, Ovary and Bier (1953) described an Arthus-like reaction apparent immediately after I/V antigen challenge, but requiring 2 to 3 hours for its full development. A similar complement dependent delayed reaction was also reported by Osler, et al., (1957) after PCA testing of antipneumococcal horse serum in experimental rats using a 2 hour sensitization period.

Later, Lavergne Raynaud and Iscaki (1966) showed that horse IgG antidiptheria antibodies mediated a

PCA reaction in guinea pigs within 10 to 15 minutes of antigen challenge, but at higher antigen doses elicited an Arthus-like reaction involving both complement fixation and a localised haemorrhagic vasculitis. Of the horse IgG subclasses, IgGab and IgGc, only the former fixes complement (Klinman, Rockey and Karush, 1967), and McGuire, Crawford and Henson (1972) reported that only IgGab will mediate a PCA reaction in experimental animals.

The variability of heterocytotropic antibodies reported in horse serum may be explained by the results of Cordal and Margni (1974) showing that horses produce both precipitating and non-precipitating IgG antibodies against hapten conjugated protein antigens. Although only non-precipitating IgGab antibodies mediated a PCA reaction in experimental animals within 15 minutes of antigen challenge after a 4 hour sensitization period, precipitating antibodies of the IgGab subclass, and to a lesser extent the IgGc subclass, mediated a reversed Arthus reaction following I/V antigen challenge 15 minutes after I/D sensitization. In addition, both precipitating and non precipitating IgGab antibodies were shown to fix complement.

The evidence in the literature appears to indicate that hyperimmune horse serum contains at least two types of IgG antibody capable of mediating a reaction in heterologous skin. One is a heterocytotropic

antibody, mediating a localised Type I hypersensitivity reaction after a 4-24 hour sensitization period. The other is a non-cytotropic antibody able to complex with antigen to produce an Arthus-like reaction. The optimum sensitization interval of this latter antibody is in the order of 2-4 hours.

9.3.3. HOMOCYTOTROPIC ANTIBODIES IN THE HORSE

In Australia, Riek (1954) described a heat-labile homocytotropic antibody-like factor in the serum of horses affected with 'Queensland Itch', a dermal hypersensitivity to the biting midge Culicoides robertsi. This factor conferred P-K sensitivity on homologous skin for at least 24 hours, and for up to ten hours on heterologous (guinea pig) skin. Subsequently, Eyre (1972a) demonstrated homocytotropic-like antibodies in the sera of ponies experimentally sensitized to bovine serum. Antigen challenge of homologous skin 24 hours after sensitization with serum from these ponies resulted in a wheal-like reaction within 5 minutes which reached its maximum at 30 minutes. The maximum antibody titre in the test sera was 1:32, although Eyre (1972b) later reported homocytotropic-like antibody titres of up to 1:64 against a commercial mixed mould antigen extract in the sera of eight horses allegedly affected with bronchopulmonary mould allergy. Schatzman et al., (1973) described the sensitization of a single experimental horse to hapten-conjugated

bovine gamma globulin, and direct I/D antigen challenge of this animal resulted in an initial wheal-like reaction followed within a few hours by a localised necrotizing vasculitis which continued for at least 24 hours. The results of this direct skin test were interpreted by these authors as a combined Type I and Type III hypersensitivity. P-K testing of serum harvested from this sensitized horse demonstrated the presence of homocytotropic-like antibodies which remained detectable at skin sites for up to 18 days after sensitization. These authors showed that the P-K response persisted for up to 7 hours after antigen challenge at sites sensitized for 10 minutes and 24 hours respectively, and proposed the prolonged response in these instances was mediated by IgG type antibodies.

Recently, Baker (1978) described the presence of homocytotropic-like antibodies against Culicoides spp. antigen in the serum of a single horse affected with recurrent seasonal dermatitis, an exudative dermatitis annually affecting around 3% of the pony population in the U.K. (McCaig, 1973). P-K testing of serum from this animal using the Culicoides antigen resulted in a prolonged response which could be elicited for up to 72 hours after sensitization.

Indirect evidence for the occurrence of homocytotropic antibodies in the serum of ponies experimentally sensitized to bovine serum was reported by Burka

et al., (1976) who demonstrated 'in vitro' antigen induced release of histamine from leucocytes harvested from these ponies. Riek (1954) had earlier described increased histamine levels in whole blood and leucocyte suspensions taken from horses affected with "Queensland itch" following incubation with C. robertsi antigen.

The immunochemical nature of the homocytotropic-like antibody in horse serum has not been established. However, on the basis of published reports of systemic anaphylaxis in the horse (McGavin, Cronwall and Mia, 1972; Eyre and Lewis, 1973), McGuire, Crawford and Henson (1972) have stated that it is reasonable to assume the occurrence of an IgE homologue in horse serum.

9.4. CYTOTROPIC ANTIBODIES IN OTHER SPECIES

Reagin-like homocytotropic antibodies directed against a range of parasitic and hapten conjugated protein antigens have been identified in a number of species. Their main physiochemical and immunological properties are summarised in Table 9.1a.

The reagin-like antibodies in these species share a number of properties with human reagin, and in most

instances satisfy Vaerman's (1970) three orders of criteria to be applied in the identification of homologous proteins in different species. These are; firstly, immunological cross reactivity and amino acid homology, secondly, the association of a protein with a particular function and thirdly, a range of biological and physiological homologies.

In the dog, guinea pig, sheep and rabbit experimentally induced anti-DNP cytotropic antibodies of the IgG class have been shown to mediate a PCA reaction in heterologous skin. These heat stable, short term sensitizing antibodies are probably analogous to the non-precipitating IgGc heterocytotropic antibody demonstrated in horse serum by Cordal and Margni (1974). In addition, an experimentally induced IgG antibody capable of short-term passive sensitization of homologous skin has been identified in mouse, rabbit, guinea pig and sheep serum. This non-reaginic homocytotropic antibody shares some immunochemical properties with the naturally occurring IgG-ST homocytotropic antibody in man. The main physiochemical and immunological properties of these IgG class cytotropic antibodies are summarised in Table 9.1b.

TABLE 9.1a

SUMMARY OF REAGIN-LIKE HOMOLOGOUS SKIN SENSITISING ANTIBODIES AS DESCRIBED IN A NUMBER OF SPECIES, INCLUDING MAN

SPECIES	SEDIMENTATION COEFFICIENT	HEAT LABILITY	THIOL REDUCTION SUSCEPTIBILITY	OPTIMUM SENSITISATION TIME (hrs)	PERSISTENCE IN SKIN (days)	COMPLEMENT FIXATION	ANTIGENIC CROSS-REACTION WITH HUMAN IgE	ELECTROPHORETIC MOBILITY	REFERENCES
Mouse	7-19 S	+	+	72	40-60	n.a.	-	n.a.	Clausen, Munoz & Bergman (1969) Schwartz & Levine (1973)
Rabbit	7-7.5 S	+	+	24-168	n.a.	-	n.a.	γ_1	Zvaifler & Robinson (1969) Stax & Ovary (1976) Ishizaka, Ishizaka and Hornbrook (1970)
Guinea pig	7-19 S	+	+	24- 48	>7	-	n.a.	β	Margni & Hajos (1973 b & c) Levine, Change and Nelson (1971)
Rat	8 S	+	+	n.a.	>31	-	+	β	Bloch & Ohman (1971) Kanyerezi, Jaton & Bloch (1971)
Sheep	7 S	+	n.a.	n.a.	14	n.a.	n.a.	γ_1	Hogarth-Scott (1969) Hudson, Bundy & Kitts (1971)
Pig	7-19 S	+	+	36- 48	14	n.a.	+	β-γ_1	Barrett(1972)
Cow	7-19 S	+	+	48- 72	>56	n.a.	+	n.a.	Hammer, Kickhofen & Schmid (1971) Doyle (1973) Wells & Eyre (1972)
Dog	8-19 S	+	+	24- 72	>15	n.a.	+	γ_1	Schwartzman, Rockey & Halliwell (1971) Halliwell, Schwartzman & Rockey (1972)
Man	8.2	+	+	24- 72	>40	-		γ_1	Ishizaka & Ishizaka (1975)

n.a. = not ascertained

TABLE 9.1b.

SUMMARY OF IgG CYTOTROPIC ANTIBODIES AS DESCRIBED IN A NUMBER OF SPECIES, INCLUDING THE HORSE AND MAN

SPECIES	TISSUE SENSITISED	OPTIMUM SENSITISATION TIME (hrs)	PERSISTENCE IN SKIN (days)	COMPLEMENT FIXATION	PASSIVE HAEMAGGLUTINATION	HEAT LABILITY	PRECIPITATION	SEDIMENTATION COEFFICIENT	ELECTROPHORETIC MOBILITY	REFERENCES
Guinea pig	Homologous	2-4	1-2	-	+	-	+/-*	7 S	γ_1	Stanworth (1973) Margni & Hajos (1973a)
	Heterologous	2-4	1-2	+	+	-	+/-*	7 S	γ_2	
Sheep	Homologous	2-4	2	-	+	-	n.a.	7 S	γ_2	Esteves, Sant'Anna, Santos Annes & Binaghi (1974)
	Heterologous	2-4	4	+	+	-	n.a.	7 S	γ_1	
Mouse	Homologous	2	minimal	n.a.	n.a.	-	n.a.	7 S	γ_2	Clausen, Munoz & Bergman (1969) Schwartz & Levine (1973)
Dog	Heterologous	2-4	n.a.	n.a.	n.a.	-	n.a.	7 S	n.a.	Rockey & Schwartzman (1967)
Rabbit	Heterologous	3	n.a.	-	+	-	-	7 S	γ_2	Margni & Binaghi (1972)
Rat	Homologous	2-6	<1	+	n.a.	-	n.a.	7 S	γ_2	Bloch & Ohman (1971)
Horse	Heterologous	3	<1	+	+	n.a.	-	n.a.	γ_2	Cordal & Margni (1974)
Man	Homologous	2-4	<1	+	n.a.	-	n.a.	7 S		Parish (1970) Bryant, Burns & Lazarus (1975)

* Both precipitating and non-precipitating IgG antibodies in the guinea pig mediate a PCA reaction

n.a. = not ascertained

9.5. DETECTION AND ASSAY OF HOMOCYTOTROPIC ANTIBODY ACTIVITY

9.5.1. 'IN VIVO' PASSIVE TRANSFER SYSTEMS

Both the P-K and PCA skin testing systems for the detection of homocytotropic antibodies have been outlined earlier (9.2.1.; 9.3.2.). The P-K test is indicated in experimental or diagnostic situations where the recipient is not expendable or where the I/V administration of large volumes of dye is impractical such as in the horse. The PCA test is indicated where the recipient is expendable and the subdermis may be inspected after sacrifice, or where extravasation of dye at the sensitized dermal sites is apparent in areas of non-pigmented skin in the intact animal, such as in the pig (Barratt, 1972) and calf (Wells and Eyre, 1970).

The factors influencing the accuracy of the P-K test in man have been outlined by Stanworth and Kuhns (1965), Augustin (1967) and Stanworth (1973) who described a number of sources of experimental variability. These include variation in the inherent dermal reactivity of individual recipients to identical reaginic serum-antigen test systems and variation in response associated with both the length of the sensitization period prior to antigen challenge and with the point of antigen challenge relative to the central point of the sensitized zone. Stanworth (1973) also re-emphasised the difficulties in I/D injection of a fixed volume at a constant depth earlier reported by Rappaport and

Becker (1949). Nevertheless, Stanworth and Kuhns (1965) demonstrated an approximately linear log dose response relationship between reaginic serum dilution and mean wheal area up to a limiting serum dilution of $^{1}/_{5}$, and Stanworth (1973) defended the use of the P-K test in man on the basis of its extreme sensitivity in detecting very low levels of reaginic antibody. Halliwell (1976) however doubted the reliability of the P-K test as a means of **assaying** serum reagin levels in dogs due to the extensive variation in dermal reactivity encountered among recipient animals.

In man, Rappaport and Becker (1949) calculated that the measurement of wheal area rather than diameter would double the slope of the dose-response curve, and at present the standard measurement of the P-K response is wheal area (Augustin, 1967). In the horse, both Eyre (1972a, 1972b) and Schatzman et al., (1973) measured P-K reactions by the increase in wheal diameter, whereas Baker (1978) measured the increase in skin fold thickness at the challenged site. This latter author apparently was able to record differences as small as 0.2mm in serial skin fold thickness measurements during the course of a P-K response elicited in a single horse.

In man, the responsiveness of hypersensitive individuals to direct I/D allergen challenge can vary depending on the body site used (Becker and Rappaport,

1948), although Stanworth and Kuhns (1965) showed no significant variation in P-K sensitivity between a number of sites distributed over a recipient's back. There is no reported evidence of site variation in P-K responsiveness in the horse, and the site of choice in this animal is the neck (Riek, 1954; Eyre, 1972a, 1972b) or shoulder (Schatzman et al., 1973).

9.5.2. 'IN VITRO' PASSIVE TRANSFER SYSTEMS

(i) Homologous Basophil Sensitization

Middleton and Sherman (1960) reported the release of histamine following antigen challenge of washed human leucocyte suspensions harvested from allergic individuals. Antigen mediated histamine release from normal human leucocytes after passive sensitization with allergic serum was later reported by Van Arsdel and Sells (1963). The involvement of circulating basophils in this reaction was demonstrated by Ishizaka, et al., (1971) using radiolabelled anti-IgE.

Although Stanworth (1973) demonstrated a linear log dose-response relationship between reagin concentration and the amount of allergen-induced histamine release from passively sensitized homologous leucocytes, the technique is not used routinely for reagin assay but is used primarily as a research tool in the investigation of the molecular mechanisms involved in mast cell sensitization and degranulation (Ishizaka, et al., 1978).

Antigen-induced 'in vitro' release of histamine from 'in vivo' sensitized leucotyes has been demonstrated in rabbits (Siraganian and Osler, 1970), cattle (Holroyde and Eyre, 1975) and horses (Burka et al., 1976; Kings and de Weck, 1980).

(ii) Heterologous Basophil Sensitization

Shelley (1962) reported rapidly developing morphological changes and degranulation of supravitally stained rabbit basophils after incubation with human reaginic serum and specific antigen, and subsequently the passive sensitization of rat peritoneal mast cells with human reaginic serum and their simultaneous antigen induced degranulation was described by Perelmutter and Khera (1969), Korotzer, Haddad and Lopapa (1971) and Perelmutter and Liakopoulou (1971). The heterologous mast cell sensitizing factor showed a number of physicochemical similarities to human IgE and paralleled the distribution of P-K activity in the eluate of reaginic serum after DEAE-Sephadex chromatography. Later reports measuring antigen induced histamine release from the sensitized rat mast cells (Gillman and Haddad, 1972; Renoux, et al., 1975), and using autoradiographic, immunofluorescent and transmission electronmicroscopic tracing techniques (Renoux et al., 1975; Pontefract and Perelmutter, 1976) have indicated that human IgE attaches to sites on the rat mast cell surface in a manner resembling the

characteristic polar 'capping' pattern of IgE molecules on human basophils (Sullivan et al., 1971).

However, Plaut, Lichtensten and Bloch (1973) were unable to obtain reproducible histamine release using comparable human reagin-rat mast cell systems to those used by other authors, and also were unable to prepare rat skin for reverse PCA using a human IgE myeloma protein. Furthermore, these authors demonstrated that the IgE myeloma protein, which inhibited the sensitization of human basophils by human IgE, did not inhibit the sensitization of rat mast cells.

Heterologous and homologous target cell sensitization with reaginic sera show a number of physiological differences. The short time required for heterologous reagin-target cell sensitization (Renoux et al., 1975) contrasts with the much longer period required for optimal homologous reagin-target cell sensitization in man (Levy and Osler, 1966) and the rat (Provoust-Danon et al., 1975). The maximal reversed anaphylaxis induced histamine release from human IgE sensitized rat mast cells, expressed as percentage of total available histamine, varies from 10-45 percent (Gillman and Haddad, 1972; Renoux et al., 1975) compared to 90 percent for homologous reagin-basophil leucocyte sensitization in man (Ishizaka et al., 1969). Also the amount of anti-IgE necessary for maximal histamine release is comparatively greater

in the heterologous reagin-target cell system (**Gillman** and **Haddad** , 1972; Ishizaka et al., 1969). Furthermore, Wyczolkowska and Provoust-Danon (1976) have shown that in contrast to the strong homologous reagin-basophil binding mechanism, human IgE could be removed from mouse peritoneal mast cells by a single washing, and Marshall (1975) has reported that normal human serum has a natural cytotoxic effect on rat peritoneal mast cells.

Recently Neilsen et al., (1976) and Neilsen and Wilkie (1977) have published indirect evidence of the binding of radiolabelled bovine reaginic immunoglobulin to rat peritoneal mast cells, and have reported the degranulation of sensitized mast cells after challenge with both specific antigen and anti-reaginic immunoglobulin. These authors did not appear to have encountered pronounced cytotoxic effect of normal bovine serum on rat peritoneal mast cells reported by Marshall (1975) under similar experimental conditions.

(iii) Sensitization of tissue preparations

Both the measurement of the amount of antigen induced histamine release from sensitized homologous intact or macerated tissue preparations, and the antigen induced contraction of sensitized smooth muscle preparations (the Schultz-Dale reaction) may be used as means of reagin detection and assay. The latter technique has been used to demonstrate fungal antigen induced

contraction of pulmonary vein preparations taken from horses showing a marked immediate reaction to direct intradermal injection of these antigens (Eyre, 1972b).

9.6. CONCLUSIONS FROM THE LITERATURE SURVEY

While a reagin-like homocytotropic antibody has been definitively identified in the serum of a number of mammalian species, a homologous antibody remains to be satisfactorily identified in horse serum. Initially, an experimental procedure designed to identify a reagin-like antibody in horse serum should be based upon satisfying as far as possible Vaerman's three orders of criteria for interspecies protein homology (9.4).

Homocytotropic-like antibodies against a midge antigen have been tentatively identified in the serum of a horse affected with recurrent seasonal dermatitis. Because of it's high morbidity in the U.K. this disease may provide a convenient model of equine Type I hypersensitivity for the investigation of the physiological and biological properties of a horse reagin-like antibody.

The P-K test provides a convenient and sensitive 'in vivo' method of detecting homocytotropic antibodies, but due to variation in recipient reactivity the test is limited as a means of assaying these antibodies.

Thus while the test may be useful in demonstrating the occurrence of homocytotropic antibodies in the serum of animals affected with recurrent seasonal dermatitis, any attempts to quantitate the P-K activity of such sera using more than one recipient horse may be liable to considerable error.

CHAPTER 10

THE DEMONSTRATION OF PASSIVELY TRANSFERABLE HOMOCYTOTROPIC-LIKE ANTIBODIES TO Culicoides pulicaris IN THE SERUM OF HORSES AND PONIES AFFECTED WITH RECURRENT SEASONAL DERMATITIS.

10.1. INTRODUCTION

Recurrent seasonal dermatitis (R.S.D.) in the United Kingdom is a localised, superficial exudative dermatitis affecting the mane, withers and tail areas of horses and ponies, usually during May to September (Baker, 1978). The clinical and pathological features of the disease have been described by Baker (1978) and Baker and Quinn (1978).

Reports of clinically similar dermatoses occurring among horses in many parts of the world have been reviewed by McCaig (1973) and Mellor and McCaig (1974), and a number of aetiological agents have been incriminated in this syndrome including, most commonly, Onchocercal filariasis (Dickmans, 1948). In the United Kingdom, Mellor and McCaig (1974) reported finding large numbers of Onchocerca cervicalis microfilaria in the ventral midline region of horse hides, although microfilaria were virtually absent from the dorsal midline regions usually affected with R.S.D..

In Australia and Japan, Riek (1954) and Ishihara and Ueno (1958) respectively identified a number of epidemiological factors which implicated biting insects in the aetiology of the disease. These workers demonstrated an immediate papular reaction in affected horses following I/D injection of a whole body extract of the biting midge Culicoides robertsi in Australia, and Simulium spp. in Japan. Riek described this reaction as being apparent within five

minutes, reaching a maximum over a period of 60 minutes, and as being histologically identical to the dermal reaction elicited by the bite of C. robertsi. Riek also demonstrated the presence of thermolabile homocytotropic-like antibodies in the serum of affected horses by passive dermal sensitization of experimental horses to midge antigen.

More recently, Mellor and McCaig (1974) proposed that on the basis of both geographical distribution and biting behaviour, C. pulicaris was the probable cause of R.S.D. in the United Kingdom. This was confirmed by McPherson and Campbell (1977, pers. comm.) who demonstrated positive wheal-like skin reactions in affected horses following I/D inoculation of C. pulicaris antigen, in contrast to the negative responses following I/D challenge with other Culicoides spp. extracts. Baker and Quinn (1978) however reported positive reactions in all R.S.D. affected animals tested by I/D injection of an antigen prepared from a range of Culicoides spp. In addition these authors also reported positive reactions in some R.S.D. affected animals following I/D injection of Stomoxys spp. antigen, although Baker (1978) reported that the homocytotropic-like antibodies in the serum of a single animal affected with the disease resulted in a positive P-K type response only after challenge with Culicoides spp. antigen.

The aim of the present study was to firstly

demonstrate homocytotropic antibodies in the serum of R.S.D. affected horses and ponies. Then later to select sera with these antibodies for investigation of their physicochemical, biological and immunological properties in accordance with Vaerman's first and third criteria of interspecies protein homology (9.4.). In this chapter the passive transfer of dermal sensitivity to C. pulicaris was demonstrated.

10.2. MATERIALS AND METHODS

10.2.1. SERA

During the spring of 1977 a circular was sent to veterinary practices in southern Scotland and northern England requesting clotted blood samples from horses and ponies affected with or having a history of R.S.D.. As a result, serum from nineteen individual cases were received (Table 10.1).

Animals showing clinical signs of the disease at the time of sampling are referred to as being in the active stage of the disease. Those animals having a history of the disease but showing no clinical signs at the time of sampling are referred to as being in the inactive stage of the disease.

Most samples reached the laboratory within 48 hours of collection. Approximately 20mls of clotted

TABLE 10.1.

DETAILS OF 19 CASES OF R.S.D. USED IN THE PRESENT STUDY; INDICATING THE BREED, AGE, DATE OF BLOOD SAMPLING AND STAGE OF THE DISEASE OF EACH ANIMAL.

CASE	DATE OF SAMPLING	ANIMAL TYPE	AGE	STAGE OF DISEASE
1	April 1977	Shetland Pony	n.a.*	Inactive
2	May 1977	Shetland Pony	4y.o.	Inactive
3	May 1977	Pony	6y.o.	Inactive
4	May 1977	Pony	7y.o.	Inactive
5	May 1977	Hunter	12y.o.	Inactive
6	May 1977	Pony	5y.o.	Inactive
7	May 1977	Shetland Pony	n.a.	Inactive
8	May 1977	Pony	n.a.	Active
9	May 1977	Cob	n.a.	Active
10	May 1977	Pony	n.a.	Active
11	May 1977	Welsh- X	6y.o.	Active
12	May 1977	Sheltand Pony	12y.o.	Active
13	June 1977	Shetland Pony	4y.o.	Active
14	June 1977	Shetland Pony	9y.o.	Active
15	June 1977	Shetland Pony	5y.o.	Active
16	June 1977	Pony	n.a.	Active
17	July 1977	Shetland Pony	7y.o.	Active
18	August 1977	Pony	Yearling	Active
19	September 1977	Pony	Yearling	Active

* n.a. not ascertained.

whole blood was obtained from each case and once received the serum was separated by centrifugation. Several 2ml aliquots of each serum were stored in sterile plastic bijoux bottles at -20°C until required.

10.2.2. PRAUZNITZ-KÜSTNER (P-K) TEST

The P-K test for the detection of soluble homocytotropic antibodies is based upon the production of an immediate wheal-like reaction following specific antigen challenge of homologous dermal sites sensitized with aliquots of the test solution. The test procedure used in man (Augustin, 1967) forms the basis of the test system used in the present study.

The end point of the P-K test has been defined as a wheal threshold size after which a reaction is deemed positive (Eyre, 1972a; 1972b). More usually, the course of the P-K reaction is plotted as the wheal size determined at specific time intervals after antigen challenge (Augustin, 1967; Stanworth, 1973). In each case the variable is the wheal size, which in man is usually expressed as wheal area (cm^2) (Augustin, 1967). In the horse, dermal reactions have been recorded using skin thickness (Baker, 1978; Hodgin et al., 1978) and wheal diameter (Eyre, 1972a; MacPherson, et al., 1979a), and a preliminary examination of the efficacy of the various methods of wheal measurement was carried out.

10.2.2.(i) Comparison of Methods of Wheal Measurement in Horse Skin.

(a) Method: Using a 10 x 0.45mm needle attached to a 1ml graduated syringe, 0.2ml of 0.001% ($^{w}/v$) histamine acid phosphate was injected I/D at six sites over the clipped lateral cervical area of an experimental horse. This is the threshold dose of histamine in horse skin (Eyre, 1972a). The resulting wheals were measured at 0, 20, 40 and 60 minutes respectively. After 60 minutes the wheals lost their well demarcated appearance. In all cases the reactions had dispersed within 120 minutes.

The following measurements were made:-

(a) Skin fold thickness (mm) using standard tuberculin testing calipers.

(b) The horizontal diameter of the wheal (mm) at the level of an ink spot placed at the centre of the initial intradermal bleb.

(c) Wheal area (cm^2) obtained by tracing the wheal outline on transparent cellulose sheet followed by overlaying on cm^2 graph paper.

(d) An additional extrapolated estimate of wheal area, πr^2 (cm^2), was derived from (b).

(b) Results: Immediately after I/D injection, a circular bleb of solution was apparent which increased in size over 60 minutes. Initially the wheal maintained its circular outline, but gradually became ovoid, and later pear shaped due to the development of de-

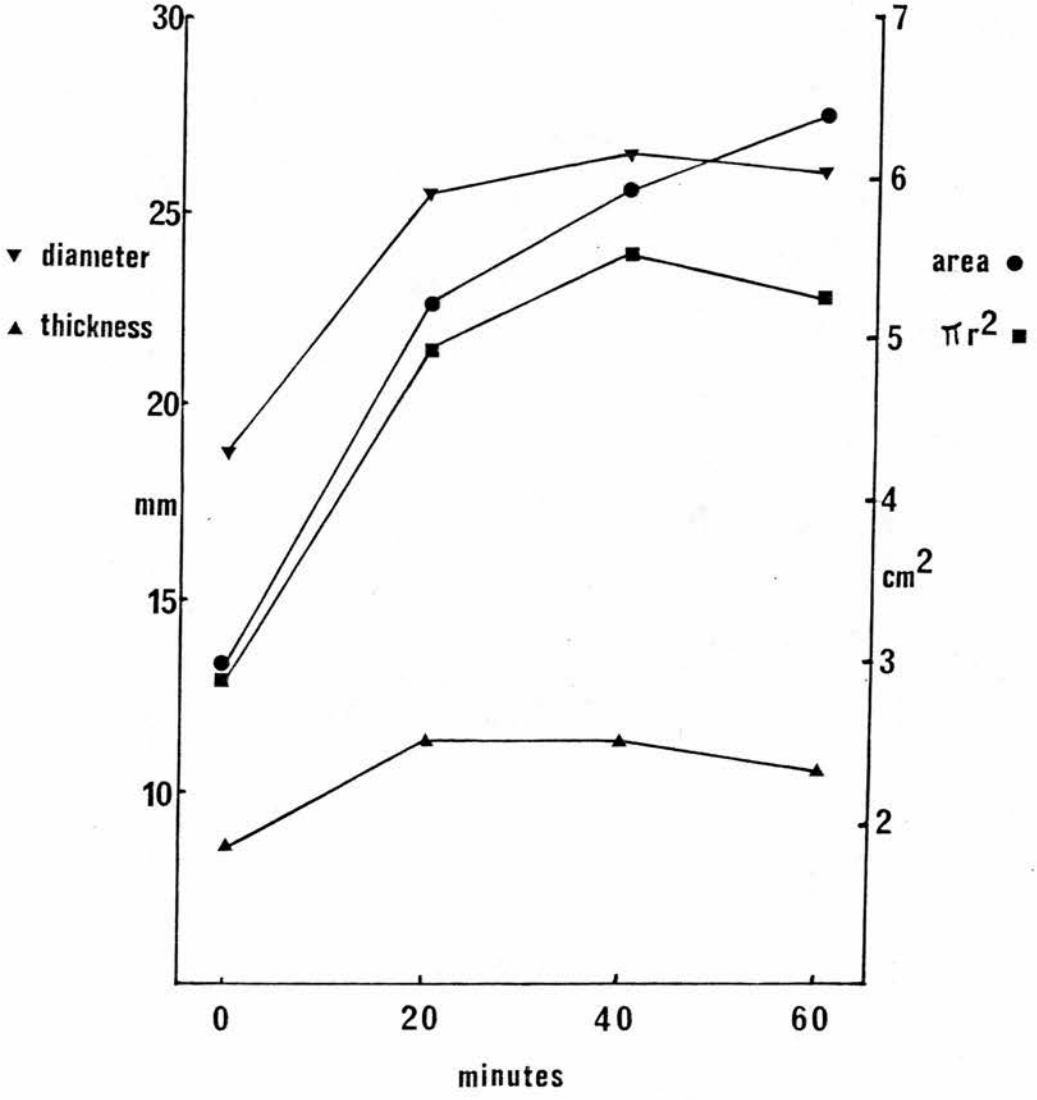


Fig. 10.1. Mean reaction size following I/D injection of histamine into horse skin.

pendant oedema. The mean reaction sizes over the first 60 minutes determined using (a), (b), (c) and (d) above are presented graphically in Figure 10.1.

Between 0 and 20 minutes after injection the mean increase in skin fold thickness of 2.41mm contrasts with the 5.94 mm and 2.34 cm^2 increases in mean wheal diameter and mean wheal area respectively. These results represent a 31% increase in mean wheal diameter in comparison with an 81% increase in mean wheal area, demonstrating the exponential increase in wheal area in relation to a linear increase in wheal diameter.

Over the initial 20 minute period the wheal retains its initial circular shape and the slope of mean wheal area parallels the slope of mean wheal πr^2. After 20 minutes the graphs diverge as a result of the development of dependant oedema.

(c) Discussion: The small increase in skin thickness recorded following the injection of histamine is comparable to the increase in skin thickness recorded following I/D injection of the non-specific mast cell degranulating compound Concanavalin-A in the horse (Hodgin, et al., 1978). However, the small changes in skin thickness which occur predispose to excessive experimental error associated with operator factors such as the pressure applied to the caliper legs and the amount of subcutaneous tissue included within the pinched skin fold.

In contrast, measurement of horizontal wheal diameter at a fixed point on the wheal surface is a simple technique with a relatively small degree of operator error. However, using cervical sites the horse's head must be held straight during the measurement, as variation in head position can distort the wheal outline.

As the wheal progressively changes in shape from circular to ovoid, graticule measurement of area provides the most accurate assessment of reaction size. However it is a prolonged procedure and consequently is difficult to perform on unwilling subjects, particularly if the wheals are painful or irritable.

In conclusion, the horizontal diameter at a fixed point on the wheal surface appears the most expedient measure of reaction size in the horse. However, P-K results were recorded initially as both wheal diameter (mm) and wheal $\pi r^2 (cm^2)$. This latter parameter was used to illustrate the exponential increase in wheal area in relation to the increase in diameter.

10.2.2.(ii) P-K Testing of serum from horses in the active and inactive stages of R.S.D.

P-K testing of horse serum using horse or pony recipients appears to have been described in the literature on only five occasions (Riek, 1954; Eyre, 1972a, 1972b; Schatzman, et al., 1973; Baker, 1978).

In each case only an incomplete account of the experimental procedure has been published. The infrequency of use of this test in horses may be due to the relatively few allergic disorders presently recognised in this species (Campbell et al., 1972). However, De Weck (1972) has stated that the exaggerated sensitivity of horse skin to relatively innocuous agents, such as physiological saline, and the expense of maintaining experimental horses both contribute towards the infrequent use of I/D procedures in this species.

The major experimental variables associated with the I/D inoculation procedure have been identified by Stanworth and Kuhns (1965) (9.5.1.), and may be minimised using a standard injection technique.

(a) Experimental animals: Recipient horses and ponies used throughout this study were obtained from experimental stock maintained by the Department of Veterinary Medicine of the University of Edinburgh. None of these animals had a known history of R.S.D.. To avoid experimental variation arising from inherent variation in recipient sensitivity each complete procedure was carried out on a single animal.

(b) Antigen Preparation: The method of McPherson and Campbell (1977, pers. comm.) based upon that described by Riek (1954) was used for the preparation of whole body extract of C. pulicaris. C. pulicaris

(Linnaeus) were identified according to Campbell and Pelham-Clinton (1960) from mixed samples of insects collected in a vacuum trap set out-doors in the vicinity of stabled horses. Although these samples had been stored at -20°C for a number of years, prolonged storage of whole midges at this temperature results in minimal deterioration of gut-blood and body proteins (Port, 1978, pers. comm.) and salivary antigen (McPherson and Campbell, 1977, pers. comm.).

A suspension of 20mg of whole midges in 5ml of sterile 0.01M PBS (pH 7.4) was macerated in a sterile Griffith tube. Following centrifugation at 3000rpm for 10 minutes the supernatant was separated from cuticular debris, passed through a 0.22µ membrane filter[1] and diluted five fold in sterile PBS. After repeated passage through a fresh 0.22µ membrane filter, 2ml aliquots of the antigen solution were stored in sterile plastic bijoux bottles at -20°C. The approximate protein concentration of the final antigen solution was 200µg/ml.

(c) Test Procedure: A test area was prepared by closely clipping the lateral cervical region of an experimental animal 1 to 2 hours prior to sensitization. A 0.1ml aliquot of serum from each case of active and inactive stage R.S.D. was injected I/D at individual

1 Millipore filters 67120, Molshiem, France.

sites over the test area using a 10 x 0.45 mm needle attached to a graduated 1ml syringe. Accurate I/D deposition of serum was indicated by the appearance of a well demarcated circular bleb of between 9 and 12mm diameter. Subcutaneous injection produced no bleb and any such sites were discarded. Individual sensitized sites were spaced 5cm or more apart and were marked by a single indelible ink spot placed at the centre of the bleb.

Twenty four hours later each site was challenged by I/D injection of 0.1ml antigen solution, placed so that the centre of the antigen bleb lay as close as possible to the ink spot.

The resulting reactions were recorded as the horizontal wheal diameter at the level of the ink spot at 0 (injection bleb), 10, 20, 30, 60, 120, and 360 minutes, and 24 hours.

Negative sensitization controls included 0.1ml each of foal serum and physiological saline. Negative challenge controls included 0.1ml each of antigen solution and sterile PBS inoculated into unsensitized skin. Augustin (1967) proposed the optional use of histamine controls to determine the variability of skin sensitivity at various sites. However, as the P-K test described in the present study was confined to a limited cervical area in a single experimental animal such controls were excluded.

10.3. RESULTS

The horizontal wheal diameters (mm) and πr^2 (cm^2) of the P-K reactions elicited by antigen challenge of homologous dermal sites sensitized with serum from cases of active and inactive stage R.S.D. are presented in Table 10.2. Figure 10.2 shows the type of reactions elicited by antigen challenge of dermal sites on a second horse sensitized with physiological saline, foal serum and active and inactive stage R.S.D. serum. Rarely, reactions to the sensitizing agent were encountered at the test site. This is shown in Figure 10.2; site 2.

No differences were observed during the initial 60 minutes between the individual inactive stage sera (cases 1-7) and both the saline and serum sensitization controls. However, while the saline sensitization and challenge controls had dispersed by 60-120 minutes, wheal-like responses persisted in cases 1 to 4 and at the serum sensitization control for up to 360 minutes, although only in case 2 was there a substantial (4mm) increase in horizontal diameter.

With the exception of cases 8, 9, 10, 11 and 15, there was a marked increase in wheal diameter over the initial 30 minutes at sites sensitized using active stage sera. This increase of between 4mm (case 19) and 7mm (case 14) represents an extrapolated increase in wheal πr^2 of between 70 and 136% respectively. Between 30 and 60 minutes the reactions in these cases

TABLE 10.2

P-K TESTING OF ACTIVE AND INACTIVE STAGE R.S.D. SERUM: HORIZONTAL WHEAL DIAMETER (mm) OF THE REACTION ELICITED BY ANTIGEN CHALLENGE OF HOMOLOGOUS DERMAL SITES PASSIVELY SENSITIZED WITH SERA FROM R.S.D. CASES LISTED IN TABLE 10.1. THE FIGURES IN PARENTHESIS ARE THE RESPECTIVE πr^2 VALUES (cm^2).

CASE \ TIME min.		0	10	20	30	60	120	360	24 HOURS
1	(I)*	12 (1.13)	12 (1.13)	12 (1.13)	14 (1.54)	11 (0.95)	12 (1.13)	14 (1.54)	D**
2	(I)	12 (1.13)	12 (1.13)	12 (1.13)	13 (1.33)	12 (1.13)	14 (1.54)	16 (2.01)	D
3	(I)	10 (0.78)	10 (0.78)	10 (0.78)	11 (0.95)	11 (0.95)	12 (1.13)	12 (1.13)	D
4	(I)	13 (1.33)	14 (1.54)	12 (1.13)	13 (1.33)	12 (1.13)	13 (1.33)	11 (0.95)	D
5	(I)	13 (1.33)	14 (1.54)	13 (1.33)	13 (1.33)	11 (0.95)	14 (1.54)	D	D
6	(I)	13 (1.33)	12 (1.13)	13 (1.33)	14 (1.54)	13 (1.33)	14 (1.54)	D	D
7	(I)	10 (0.78)	12 (1.13)	12 (1.13)	12 (1.13)	12 (1.13)	14 (1.54)	D	D
8	(A)	12 (1.13)	10 (0.78)	10 (0.78)	10 (0.78)	11 (0.95)	12 (1.13)	14 (1.54)	D
9	(A)	14 (1.54)	14 (1.54)	12 (1.13)	11 (0.95)	12 (1.13)	14 (1.54)	D	D
10	(A)	12 (1.13)	11 (0.95)	14 (1.54)	15 (1.77)	14 (1.54)	14 (1.54)	14 (1.54)	D
11	(A)	10 (0.78)	10 (0.78)	10 (0.78)	12 (1.13)	11 (0.95)	12 (1.13)	D	D
12	(A)	10 (0.78)	15 (1.77)	16 (2.01)	16 (2.01)	16 (2.01)	20 (3.14)	23 (4.15)	D
13	(A)	11 (0.95)	15 (1.77)	15 (1.77)	16 (2.01)	16 (2.01)	23 (4.15)	25 (4.91)	D
14	(A)	13 (1.33)	17 (2.27)	18 (2.55)	20 (3.14)	19 (2.84)	25 (4.91)	26 (5.31)	D
15	(A)	12 (1.13)	15 (1.77)	15 (1.77)	15 (1.77)	16 (2.01)	18 (2.55)	21 (3.46)	D
16	(A)	11 (0.95)	14 (1.54)	15 (1.77)	16 (2.01)	16 (2.01)	21 (3.46)	22 (3.80)	D
17	(A)	11 (0.95)	15 (1.77)	16 (2.01)	16 (2.01)	15 (1.77)	21 (3.46)	23 (4.15)	D
18	(A)	12 (1.13)	15 (1.77)	16 (2.01)	18 (2.55)	18 (2.55)	23 (4.15)	23 (4.15)	D
19	(A)	13 (1.33)	16 (2.01)	16 (2.01)	17 (2.27)	16 (2.01)	18 (2.55)	20 (3.14)	D
Saline Sensitization		11 (0.95)	11 (0.95)	12 (1.13)	12 (1.13)	13	D	D	D
Foal Serum Sensitization		11 (0.95)	12 (1.13)	12 (1.13)	12 (1.13)	13 (1.33)	14 (1.54)	D	D
Antigen Challenge		10 (0.78)	10 (0.78)	10 (0.78)	9 (0.63)	9 (0.63)	D	D	D
PBS Challenge		10 (0.78)	11 (0.95)	10 (0.78)	10 (0.78)	9 (0.63)	D	D	D

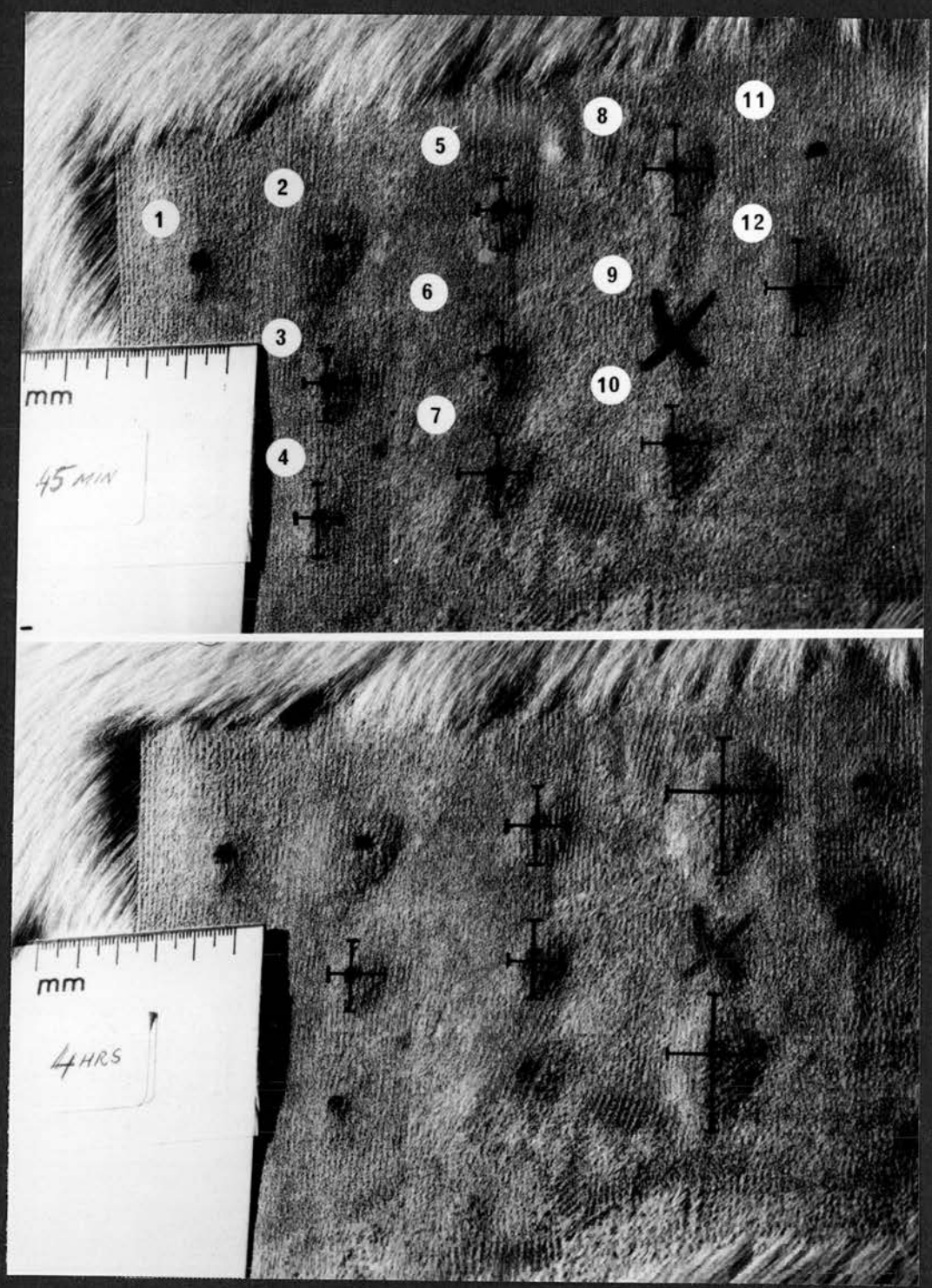
* A - Active Stage
I - Inactive Stage
**D - Dispersed.

Fig. 10.2. P-K responses in sensitized horse skin after C. pulicaris antigen challenge. Sites 1-12 were sensitized as follows:-

Site 1 (Physiological Saline)
2 Foal serum (no challenge) *
3 Foal serum
4 Inactive R.S.D. serum, case 2
5 Inactive R.S.D. serum, case 5
6 Active R.S.D. serum, case 9
7 Active R.S.D. serum, case 13
8 Active R.S.D. serum, case 14
9 Discarded site**
10 Active R.S.D. serum, case 19
11 Unsensitized
12 Active R.S.D. serum, case 17

* Non-specific reaction to sensitizing serum.

** Subcutaneous sensitization.


1
2
3
4
5
6
7
8
9
10
11
12
mm
45 MIN
mm
4 HRS

remained relatively static. However between 60 and 120 minutes there was a further increase in reaction diameter of between 2mm (case 19) and 7mm (case 14), representing an extrapolated increase in wheal πr^2 of between 37% and 106%. The increase continued in most cases for up to 360 minutes. In all cases, no reaction was present at 24 hours.

Active stage sera from cases 8 to 11 showed no increasing reaction, and in these cases the reaction was essentially similar to both the inactive stage sera and serum sensitization control. Case 15 showed only a slight increase in reaction (3mm) at 30 minutes, but increased markedly up to 360 minutes and had dispersed at 24 hours.

The mean $\pm$ 1 S.D. of the wheal horizontal diameter (mm) and πr^2 (cm^2) at 0, 30, 60, 120 and 360 minutes after I/D antigen challenge of sites sensitized with serum from inactive stage R.S.D. cases (1-7) and active stage R.S.D. cases (12-19) are presented in Table 10.3.

MacPherson et al., (1979a) deemed positive a dermal response elicited by direct antigen challenge of horse skin whose horizontal diameter exceeded the mean $\pm$ 2 S.D. of the control reactions. In the present study of P-K responses in horse skin, with the exception of case 15 at 30 minutes the responses up to 360 minutes after antigen challenge at sites sensitized

TABLE 10.3.

MEAN $\pm$ 1 S.D. OF THE HORIZONTAL DIAMETERS (mm) AND πr^2 (cm^2) OF THE REACTIONS INDUCED BY I/D ANTIGEN CHALLENGE OF SITES SENSITIZED WITH SERUM FROM INACTIVE STAGE R.S.D. CASES 1-7 AND ACTIVE STAGE R.S.D. CASES 12-19.

TIME (mins.)	ACTIVE STAGE R.S.D. CASES 12-19		INACTIVE STAGE R.S.D. CASES 1-7.	
	diameter $\bar{x}$ S.D.	πr^2 $\bar{x}$ S.D.	diameter $\bar{x}$ S.D.	πr^2 $\bar{x}$ S.D.
0	11.6 $\pm$ 1.07	1.07 $\pm$ 0.20	11.9 $\pm$ 1.35	1.12 $\pm$ 0.25
30	16.7 $\pm$ 1.58	2.22 $\pm$ 0.44	12.9 $\pm$ 1.07	1.31 $\pm$ 0.21
60	16.5 $\pm$ 1.31	2.15 $\pm$ 0.36	11.7 $\pm$ 0.76	1.08 $\pm$ 0.14
120	21.1 $\pm$ 2.47	3.55 $\pm$ 0.82	13.3 $\pm$ 0.95	1.39 $\pm$ 0.20
360	22.9 $\pm$ 1.96	4.13 $\pm$ 0.71	D*	D*

* D = Dispersed.

with sera from cases 12-19 were in excess of the mean + 2 S.D. of both the horizontal diameter and πr^2 of the responses elicited at sites sensitized with sera from cases 1 - 7. The static wheal-like reactions at the serum sensitization control site and the inactive stage R.S.D. serum sensitization sites (cases 1 - 7) were deemed negative. The reactions elicited at sites sensitized with active stage R.S.D. serum (cases 12-19) were in excess of the upper limit ($\bar{x}$ + 2 S.D.) applied to the negative reactions and were deemed positive. The reactions at sites sensitized with sera from active R.S.D. cases 8 - 11 do not exceed the $\bar{x}$ + 2 S.D. limit and were deemed negative.

The changes in mean πr^2 of the positive responses (cases 12 - 19) and the negative responses (cases 1 - 7) up to 360 minutes after antigen challenge are shown graphically in Figure 10.3 along with the $\bar{x}$+2 S.D. limit applied to the latter results.

Morphologically the positive reactions elicited at sites sensitized with active stage serum (Fig. 10.2, sites 8 and 10) appeared as well demarcated wheal-like areas which increased radially in size from the initial injection bleb. Between 30 and 60 minutes the wheals lost their circular outline and became ovoid. Between 120 and 360 minutes after challenge the wheals became painful and in some instances developed dependant oedema. Negative responses (Fig. 10.2; sites 3, 4, 5 and 6) did not develop a well demarcated appearance

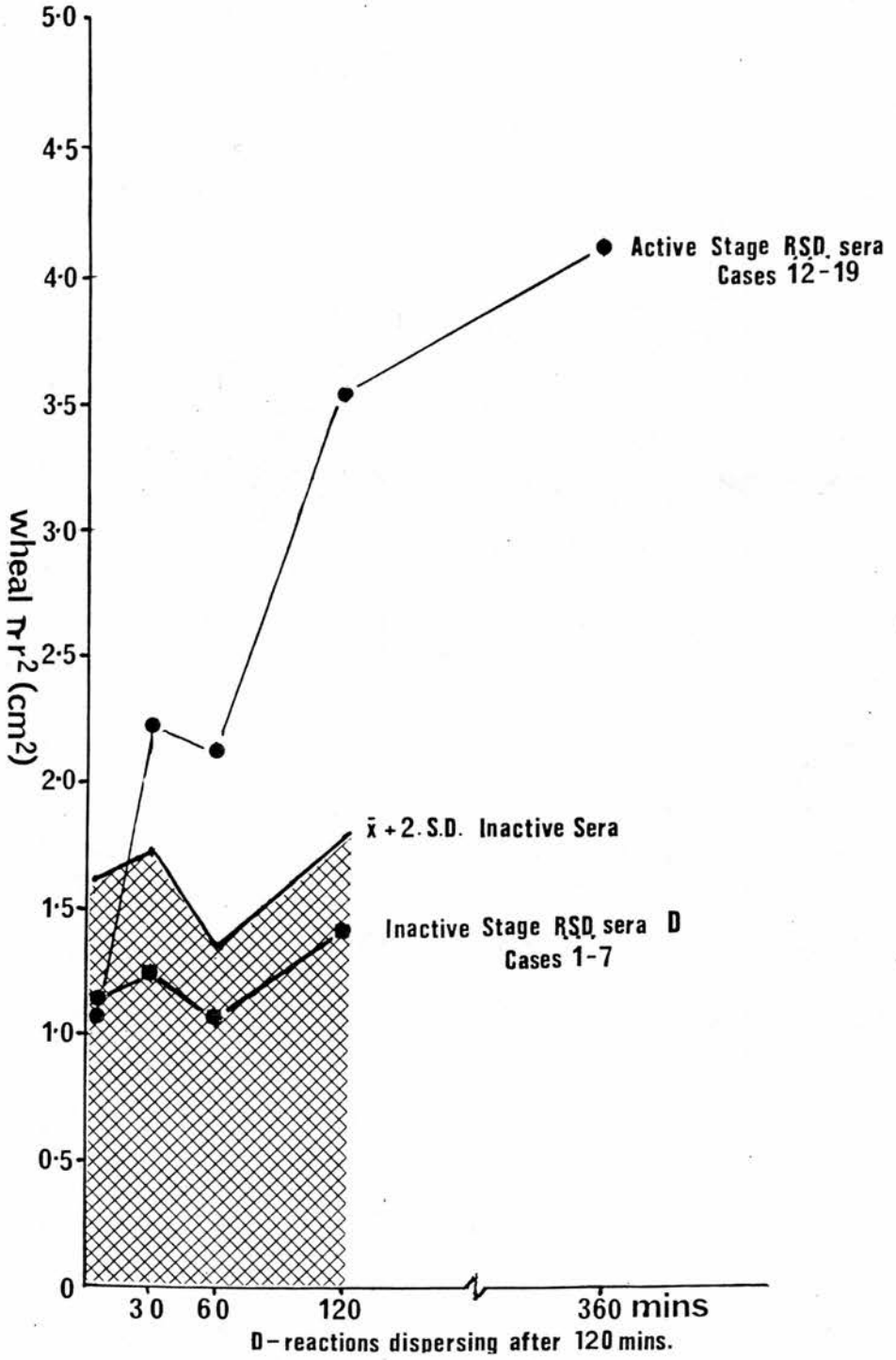


Fig. 10.3. Mean πr^2 of the P-K responses elicited by C. pulicaris antigen challenge of sites sensitized with active R.S.D. sera (cases 12-19; positive responses) and inactive R.S.D. sera (cases 1-7; negative responses) and the $\bar{x}$ + 2 S.D. limits applied to the latter results.

and persisted as static or slowly enlarging dermal swellings.

Prolonged storage of serum at -20°C appeared to result in the loss of the well demarcated 4-6 hour responses at some sites sensitized with active stage R.S.D. sera (Fig. 10.2; sites 7 and 12). In these instances a diffusely raised dermal area was apparent at this time.

10.4. DISCUSSION

The occurrence of immediately positive P-K responses at sites sensitized with active stage R.S.D. sera after challenge with C. pulicaris extract indicates the presence of homocytotropic-like antibodies to the midge antigen in the sera of affected animals. This is in accordance with the earlier observations of Baker (1978) on a single case of R.S.D., and of Riek (1954) on a number of horses naturally hypersensitized to bite of the midge C. robertsi.

The magnitude of positive P-K responses observed are similar to those resulting from both direct I/D challenge of sensitized horses with fungal antigens (MacPherson, et al., 1979a; Halliwell, et al., 1979) and from P-K testing of sensitized horse sera and Parascaris equorum antigen (Mansman and Mansman, 1975). The persistance of swellings at negative

test or negative control sites for up to 6 hours has been reported during both P-K and direct I/D testing of horses (Schatzman, et al., 1973; MacPherson, et al., 1979a), and probably reflects the extreme sensitivity of horse skin to relatively innocuous challenge noted by De Weck (1972).

In the present series of observations on P-K responses, positive responses were not invariably elicited at sites sensitized with active stage R.S.D. serum, and negative responses were observed in four instances where the donor animals were actively affected with the disease. In these cases, the sera was collected during April and early May, when C. pulicaris is just beginning to become active in the U.K. (Campbell and Pelham-Clinton, 1960; Mellor and McCaig, 1974), suggesting that the absence of detectable levels of circulating homocytotropic-like antibody in these cases may result from the absence of significant antigenic stimulation. Although no reports of challenge associated variation in circulating homocytotropic antibody levels in horses have appeared in the literature, Lichtenstein, Norman and Connel (1967) and Berg and Johansson (1971) have demonstrated marked increases in P-K titres and circulating IgE levels respectively in pollen sensitive human patients with the onset of allergen exposure.

Alternatively, the absence of detectable responses during P-K testing of some active stage R.S.D. sera may

have resulted from the presence of circulating antigen-specific IgG type 'blocking' antibodies associated with antigen exposure during the previous summer. Antigen specific IgG antibodies have been induced in man following prolonged allergen exposure (Cooke, et al., 1935; Devey, Wilson and Wheeler, 1976), and Stanworth (1973) has stated that they may competitively interfere with reagin-allergen interaction during the P-K test. However, the biological role of blocking antibodies in the direct inhibition of allergen binding is questionable as Ishizaka and Ishizaka (1973) showed that decreased serum IgE levels occur with increasing allergen-specific IgG levels, although the mechanism and clinical significance of this negative modulation have not been fully elucidated. The occurrence of similar blocking antibodies in the horse has not been investigated. However, Devey et al., (1976) have shown that blocking antibodies in man belong predominantly to the IgG_4 subclass, and Fisher and Connell (1962) had earlier shown that blocking antibodies are able to mediate typical PCA reactions in heterologous skin. Thus they may be analogous to the non-precipitating IgGab antibodies identified by Cordal and Margni (1974) in experimentally produced hyper-immune horse serum (9.3.1). These IgGab antibodies characteristically mediate a PCA reaction in heterologous skin after a 4 hour sensitization interval, and Riek (1954) described P-K

responses elicited in guinea pig skin by C. robertsi antigen challenge 6-10 hours after sensitization with serum from horses affected with Queensland Itch. However Riek reported this heterocytotropic activity of horse serum to be thermolabile, suggesting it is independent of IgG type antibodies. Nevertheless the occurrence of circulating heterocytotropic-like antibodies in horses affected with R.S.D. and their possible blocking effect upon reaginic sensitization merits further investigation, particularly from the viewpoint of immunoprophylaxis of the disease.

The P-K response elicited by antigen challenge of sites sensitized with serum from R.S.D. cases 12-19 appears biphasic. The first phase of the dermal response reaches a maximum within 30 minutes and is followed after a short period, during which the reaction remains static, by a second phase during which the wheal increases in size up to around 6 hours after challenge. In all cases no reaction was present at 24 hours. A similar prolonged P-K reaction was reported by Baker (1978) using serum from a single case of R.S.D.. Riek (1954) however described P-K responses obtained using the naturally acquired C. robertsi hypersensitivity model as being positive within 60 minutes, but did not report observations on these responses after this time. Eyre (1972a) used serum from ponies experimentally sensitized to bovine serum and described P-K reactions in horse

skin which maximized at 30 minutes although he also did not report observations after this time. Schatzman et al., (1973) however reported a P-K reaction which increased in size for up to 7 hours after sensitization with serum from a horse experimentally immunized to hapten-conjugated bovine gamma globulin. Mansman and Mansman (1975) have stated that direct I/D challenge of horse skin with Parascaris equorum and Gasterophilus spp. extracts and the artificial mast cell degranulators compound 48/80 and D-tubocurarine both result in an immediate wheal-like reaction with 30 minutes which persist for up to 4 hours.

These prolonged dermal reactions elicited by both parasitic antigens and chemical mast cell degranulators suggest that the Type I or basophil dependant hypersensitivity response in horse skin may have a delayed component, unlike the classical wheal and flare response in man. This response in the horse is morphologically similar to the IgE dependent late cutaneous anaphylactic response (LCAR) in man, which is typically elicited in allergic individuals by direct I/D challenge with higher allergen doses than those necessary to elicit a transient wheal and flare response (Umemoto et al., 1976). Similar though less intense LCARs have been observed during P-K testing of human reaginic serum in homologous skin (Dolovich et al., 1973; Solley, et al., 1976). The LCAR in man

appears allergen dose dependent and the C. pulicaris antigen dose used to elicit P-K responses in the present study was approximately ten times that of the C. robertsi antigen dose used by Riek (1954).

Baker (1978) was able to elicit a prolonged or biphasic response by antigen challenge of homologous skin sites up to 72 hours after sensitization with R.S.D. serum. Schatzman et al., (1973) however were able to elicit an immediate P-K response for up to 18 days after sensitization but could elicit a prolonged reponse only up to 24 hours after initial sensitization. These latter authors concluded that the delayed phase of the respone was an Arthus reaction mediated by passively transferable, non-homocytotropic precipitating antibodies. Yet the P-K response described by these authors was morphologically and temporally unlike the necrotizing response elicited by direct I/D challenge of their experimentally sensitized horse, which they also identified as an Arthus response.

The possibility remains that the second or delayed phase of the P-K response in this study may be an Arthus response. This is reinforced by the observation of Riek (1954) who described transient wheal-like reactions after I/D antigen challenge of horses hypersensitized to C. robertsi, but after challenging these horses with higher antigen doses he reported severe necrotizing reactions lasting up to 48 hours. Similarly, Baker and Quinn (1978) described severe reactions persisting beyond 24 hours

after direct I/D challenge of R.S.D. affected horses with Culicoides extract. These observations suggest the participation of an IgG mediated Arthus component in the reactions elicited in actively challenged horses. However the IgE dependent LCARs in man have been reported to persist for up to 48 hours after direct antigen challenge and have been shown to occur independently of the deposition of immune complexes (Solley et al., 1976).

In conclusion, the results show that homocytotropic-like antibodies capable of sensitizing homologous skin to C. pulicaris antigen for up to 24 hours may be demonstrated in the serum of animals affected with R.S.D.. Their presence suggests that the immunopathogenesis of this disease involves a Type I hypersensitivity component. Furthermore, the P-K response to antigen challenge of homologous dermal sites sensitized with serum harvested from active cases of R.S.D. is biphasic. However the respective roles of passively transferable homocytotropic and non-homocytotropic antibodies in the genesis of this response has not been established.

CHAPTER 11

SOME BIOLOGICAL AND PHYSICOCHEMICAL PROPERTIES OF AN EQUINE HOMOCYTOTROPIC-LIKE ANTIBODY.

11.1. INTRODUCTION

The major biological and physicochemical properties of the reagin-like homocytotropic antibodies identified in a number of mammalian species have been summarised in Table 9.1a. In general, these antibodies are heat labile, are susceptible to thiol reduction, have a sedimentation coefficient of between 7 and 19S and a slow anodal electrophoretic mobility on zone electrophoresis. These antibodies remain fixed in homologous skin for longer than 7 days and have an optimum sensitization interval of between 24 and 72 hours. In addition, human (Ishizaka and Ishizaka, 1966), canine (Halliwell, et al., 1975) and bovine (Hammer, et al., 1971) homocytotropic antibodies are eluted from DEAE cellulose ion exchange resin over a molar range of 0.025M-0.035M, 0.025M-0.05M and 0.025-0.03M phosphate buffer (pH 8.0) respectively, indicating significant similarities in net molecular charge.

In this chapter, some biological and physicochemical characteristics of the homocytotropic-like antibody in the serum of horses and ponies affected with R.S.D. were examined and were compared with the characteristics of the reagin-like antibodies of other species in accordance with Vaerman's third criterion of interspecies protein homology.

11.2. HEAT AND THIOL SENSITIVITY OF EQUINE HOMOCYTOTROPIC-LIKE ANTIBODIES

11.2.1. INTRODUCTION

The irreversible loss of homocytotropic activity in human serum resulting from heating to 56°C has been known for some time (Coca and Grove, 1925), and in 1954 Riek demonstrated the loss of P-K activity from horse serum after heating to 56°C for 30 minutes.

Thermolability is a characteristic of mammalian reagin-like antibodies (Table 9.1a), and the results of a stereochemical study of the effect of heat on the molecular structure of human IgE by Dorrington and Bennich (1973) showed that thermal sensitivity is probably restricted to the cell-binding CE3 and/or CE4 domains in the -COOH terminal of the epsilon chain. In contrast IgG homocytotropic antibodies are stable at 56°C (Table 9.1b).

The characteristic thiol sensitivity of human homocytotropic antibodies was reported by Leddy et al. (1962) who concluded that the degree of inactivation is dependent upon the initial reagin titre, the activity of the reducing agent and the duration of thiol-serum incubation. Mild thiol reduction (0.05M - 0.1M 2-Mercaptoethanol) of human IgE appears to result in the irreversible loss of cytotropic activity following cleavage of both inter and intra-epsilon chain disulphide bonds (Stanworth, et al., 1970;

Takatsu, Ishizaka and Ishizaka, 1975). However, loss of cytotropic activity may occur without cleavage of the disulphide bonds in the crucial CE3 and CE4 domains, leading both Takatsu et al., (1975) and Bennich et al., (1976) to propose that the conformation of the Fab fragment may influence the activity of the cell binding site on the Fc fragment. In contrast, IgG antibodies dialysed against 0.1M 2-Mercaptoethanol at near neutral pH characteristically retain their biological activity (Rockey and Kunkel, 1962; Stanworth and Turner, 1973), and equine IgG antibodies appear typical in this respect (Podliachouk and Kaminski, 1976).

11.2.2. MATERIALS AND METHODS

To determine the effect of heat and thiol reduction on the equine homocytotropic-like antibody, the P-K activity of pooled P-K positive R.S.D. sera (Cases 13, 14 and 19) was examined before and after the following treatments;

(i) A 0.5ml aliquot of the pooled serum was heated at 56°C for 2 hours in a water bath.

(ii) A 0.5ml aliquot of pooled serum was dialysed against 0.1M 2-Mercaptoethanol[1] (2-ME) in 0.01M PBS (pH 7.4) for 4 hours at room temperature followed by dialysis at room temperature for a further 4 hours against 500ml 0.02M iodoacetamide[1] in PBS. The reduced and alkylated serum was then dialysed against several changes of 500ml PBS for 24 hours at 4°C.

1 Sigma Chemical Co., Poole, England.

(iii) A 0.5ml aliquot of pooled serum was dialysed against 500ml 0.02M iodoacetamide in PBS for 4 hours at room temperature followed by dialysis against several changes of 500ml PBS for 24 hours at 4°C.

Routine agarose electrophoresis (3.2.1.) of the pooled untreated serum and the 2-ME reduced and iodoacetamide treated control sera was carried out to examine the effect of reduction on the electrophoretic profile for comparison with the electrophoretic observations on thiol reduced human serum reported by Leddy et al., (1962).

11.2.3. RESULTS

Following dialysis against 0.02M iodoacetamide and PBS only, no change occurred in the electrophoretic profile of the pooled serum. However, following dialysis against 0.1M 2-ME and iodoacetamide a relative increase in the alpha-2, beta and gamma-1 components was observed, concurring with the observations of Leddy et al., (1962) on human serum.

The results of P-K testing of pooled R.S.D. serum before and after heat and controlled thiol reduction are presented in Table 11.1. Antigen challenge of the P-K positive serum sensitized site resulted in a positive biphasic response in contrast to the negative response elicted at both the sensitization and challenge control sites. Heating and thiol reduction alkylation of the P-K positive serum resulted in elimination of the biphasic

TABLE 11.1.

THE EFFECTS OF HEAT AND 2-ME REDUCTION ON THE P-K ACTIVITY OF POOLED P-K POSITIVE R.S.D. SERUM. P-K ACTIVITY IS EXPRESSED AS THE WHEAL HORIZONTAL DIAMETER (mm) AT 0, 30, 60, 120 AND 360 MINUTES AND 24 HOURS AFTER C. PULICARIS ANTIGEN CHALLENGE.

Treatment	TIME AFTER ANTIGEN CHALLENGE (MINS.)					24 hours
	0	30	60	120	360	
Untreated	10	16	16	20	21	D*
56°C for 2 hours	11	11	12	14	14	D
2-ME + iodoacetamide	12	14	14	15	D	D
iodoacetamide alone	11	15	18	22	22	D
A	10	11	10	12	12	D
B	10	12	D	D	D	D
C	9	9	10	D	D	D

A - Foal serum sensitization control.

B - Physiological saline sensitization control.

C - Antigen challenge control.

* D - Dispersed.

response. Alkylation alone had no effect on the response.

11.2.4. DISCUSSION

In common with the reaginic antibody in a number of other species the homocytotropic-like antibody in the serum of R.S.D. affected animals is susceptible to molecular dissociation by 2.ME. The conditions of thiol reduction employed in present series are milder than those reported to destroy the homocytotropic antibody activity of bovine serum (Hammer et al., 1971; Wells and Eyre, 1972) and canine serum (Rockey and Schwartzman, 1967), although considerable variation exists throughout the literature on the optimal experimental conditions necessary for thiol reduction of reagin-like antibodies. The abolition of both the immediate and delayed components of the P-K response following thiol reduction suggests that both phases represent an integral part of a single response. However, should the delayed response be dependent upon an initiating immediate response the experimental results cannot wholly exclude the participation of thiol stable IgG antibodies in the genesis of the delayed response.

The loss of P-K activity of pooled R.S.D. serum following heating to 56°C for 4 hours confirms the earlier observation of Riek (1954) on the thermolability of the equine homocytotropic-like antibody.

11.3. PERSISTENCE OF EQUINE HOMOCYTOTROPIC-LIKE ANTIBODIES IN HOMOLOGOUS SKIN.

11.3.1. INTRODUCTION

The literature on the persistence of mammalian reagin-like antibodies in homologous skin has been summarised in Table 9.1a. In the horse, the limited data has shown that homocytotropic-like antibodies are detectable for between 3 (Baker, 1978) and 18 (Schatzman et al., 1973) days after sensitization. In both these reports however serum from a single source was tested, and in the latter report 5 individual recipients were sensitized and each challenged only once after an increasing interval. This introduces potential variation in P-K reactivity between recipients into the experiment, a factor known to be a source of experimental variation in man (Stanworth and Kuhns, 1965), and the dog (Halliwell et al., 1975).

11.3.2. MATERIALS AND METHODS

Six dermal sites on the lateral cervical region of a single experimental horse were sensitized with 0.1ml pooled P-K positive R.S.D. sera (cases 13, 14 and 19). These sites were each challenged with 0.1ml C. pulicaris antigen at either 2, 4, 24, 48 hours, and 5 and 9 days after sensitization, and the P-K response recorded. Foal serum and physiological saline sensitization controls and an antigen challenge control were included at each test site.

11.3.3. RESULTS

The P-K reactions elicited by antigen challenge

TABLE 11.2.

P-K RESPONSE ELICITED IN HOMOLOGOUS SKIN AFTER SENSITIZATION WITH POOLED P-K POSITIVE R.S.D. SERUM. THE RESPONSE IS EXPRESSED AS HORIZONTAL WHEAL DIAMETER (mm) AT 0, 30, 60, 120 AND 360 MINUTES AND 24 HOURS AFTER C. PULICARIS ANTIGEN CHALLENGE.

A. Pooled P-K positive R.S.D. sera.
B. Foal serum sensitization control.
C. Physiological saline sensitization control.
D. Antigen challenge control.

Time after sensitization		TIME AFTER ANTIGEN CHALLENGE (MINS.) 0	30	60	120	360	24 hours
2 hours *	A	11	18	18	20	25	D**
	B	14	16	13	16	12	D
	C	10	10	10	D	D	D
	D	8	8	D	D	D	D
4 hours *	A	11	18	17	18	24	D
	B	15	15	14	14	D	D
	C	10	10	D	D	D	D
	D	8	D	D	D	D	D
24 hours	A	10	16	16	20	21	D
	B	11	11	10	12	12	D
	C	10	12	10	14	14	D
	D	10	0	0	5	8	D
48 hours	A	10	20	20	27	32	D
	B	10	12	12	15	15	D
	C	10	12	12	15	16	D
	D	10	12	12	15	16	D
5 days	A	10	15	16	22	22	D
	B	10	12	12	15	D	D
	C	10	12	12	15	D	D
	D	10	12	12	15	D	D
9 days	A	10	12	12	12	14	D
	B	10	10	10	10	12	D
	C	10	10	10	12	14	D
	D	10	10	10	10	12	D

* Antigen is innoculated into sensitization bleb.

** D - Dispersed.

at increasing time intervals after sensitization are presented in Table 11.2.

The results demonstrate that in this study homocytotropic-like antibodies were detected up to 5 days after sensitization. Furthermore, I/D antigen challenge up to 5 days post-sensitization resulted in a biphasic response.

11.3.4. DISCUSSION

The prolonged persistance of passively transferable P-K mediating antibodies in homologous skin indicates that these antibodies are able to bind to cells within the dermis and are thus true homocytotropic antibodies.

The duration of detectable levels of these antibodies in the present series, 5-9 days, is less than the 18 days reported by Schatzman et al., (1973) using hyperimmune horse anti-bovine gamma globulin serum. However, homocytotropic antibody activity in human skin has been shown to decay exponentially with a half life of 13 days (Augustin, 1967), corresponding to the half life of 8.5-14 days of radiolabelled IgE in human skin (Ishizaka and Ishizaka, 1971). This indicates that the duration of detectable levels of homocytotropic antibody in skin is dependant upon the initial antibody titre, and some variation between different test models in the same species is to be expected.

Although the presence of a biphasic P-K response

up to 5 days post sensitization concurs with the observations of Baker (1978) using a similar experimental model, they differ from those of Schatzman et al., (1973) who elicited a biphasic response only up to 24 hours after sensitization. Subsequent challenge of sensitized sites by these latter authors resulted in only an isolated immediate response, and on this basis they concluded that the delayed response represented an Arthus-type reaction mediated by non-cytotropic antibodies. However, by the same rationale the delayed component of the P-K response observed both in the present study and by Baker (1978) 5 and 3 days after sensitization respectively is unlikely to be an Arthus-type response due to the rapid physiological diffusion of non-fixed protein into interstitial fluid and its removal via the circulation. Although the disappearance rate of homologous non-cytotropic IgG antibodies from skin sites in the horse has not been established, the initial half-life of free IgG in human skin has been shown to be approximately 12 hours (Kuhns, 1961; Ishizaka and Ishizaka, 1971), with only some 2 percent of the original levels remaining 5 days after inoculation (Ishizaka and Ishizaka 1971). In addition, Augustin (1967) has described an 8 hour half-life of non-cytotropic rabbit IgG in guinea pig dermis, and passive dermal Arthus responses elicited in laboratory animals using hyperimmune horse serum

(9.3.2.) have indicated a similar dermal half-life of horse IgG in these species.

Thus the prolonged P-K response observed in horse skin appears dependent upon the homocytotropic antibodies and is similar in this respect to the IgE dependent late cutaneous anaphylactic response in man.

11.4. THE ELUTION OF EQUINE HOMOCYTOTROPIC ANTIBODY ON GEL FILTRATION AND ION EXCHANGE CHROMATOGRAPHY OF P-K POSITIVE R.S.D. SERUM

The elution of a protein from gel filtration or ion exchange media is principally dependent upon molecular size and net molecular charge respectively (Fahey and Terry, 1973), the latter being determined by the intramolecular distribution of charge carrying amino acid groups. Thus similarily in elution characteristics between proteins is indicative of homology in molecular size and net charge. In this series of experiments the elution characteristics of equine homocytotropic antibody was determined for comparison with those of the reaginic antibodies in other species.

In addition, Stanworth (1965) has stressed the importance of identical elution characteristics in attributing a biological function to a particular protein, and the pattern of elution of P-K activity

of R.S.D. serum from gel filtration chromatography and ion exchange chromatography was compared with those of the major immunoglobulin classes IgG, IgG(T) and IgM.

11.4.1. GEL FILTRATION (EXCLUSION) CHROMATOGRAPHY

(i) Introduction

Early studies on gel filtration chromatography of human reaginic serum examined the distribution of reaginic antibody in the eluate in relation to the distribution of known serum components, in particular IgA (Fireman, Vannier and Goodman, 1963). Subsequently the fractionation method became an integral part of the isolation and final identification of IgE (Ishizaka et al., 1966b; Ishizaka and Ishizaka, 1967). On Sephadex G200 chromatography, the bulk of the 8S human IgE was eluted in the ascending portion of the 7S peak (Johansson et al., 1968), and a similar distribution of reaginic antibody on Sephadex G200 fractionation has been demonstrated using canine (Halliwell et al., 1975), bovine (Hammer et al., 1971), and pig (Barratt, 1972) serum.

(ii) Materials and Methods

Sephadex G200[1] slurry was prepared in 0.01M PBS (pH 74) according to the manufacturer's instructions, and packed into a 2.5 cm x 40 cm glass column by gravity flow. A 3.5 ml aliquot of pooled

1 Pharmacia Fine Chemicals, Uppsala, Sweden.

P-K positive R.S.D. serum was applied to the column using an upward flow adaptor and peristaltic pump, providing a flow rate of 27ml per hour PBS. Monitoring and collection of the eluate was carried out as previously described (7.2.2).

A total of eight final protein fractions were collected by pooling of the protein containing eluate. These fractions were concentrated by evaporation to the approximate protein concentration of whole serum (65g/L) as suggested by Stanworth (1973). However, concentration of the fractions was limited by the final volume, 0.5ml being the minimum workable volume.

Following concentration, the fractions were dialysed against repeated changes of PBS for 48 hours at 4°C. The volume and protein concentration (g/L) of the collected G200 fractions 1-8 after concentration and dialysis are presented in Table 11.3.

After passage of the fractions through a 0.22μ membrane filter[1], 0.1ml was injected I/D into cervical sites on an experimental horse, to be followed 24 hours later by antigen challenge. Prefractionation R.S.D. serum was included as a positive sensitization control along with PBS and foal serum negative sensitization controls.

(iii) Results

Following I/D inoculation of the Sephadex G200 fractions, a localised painful reaction was

1 Millipore Corporation, 67120 Molshiem, France.

TABLE 11.3.

VOLUME AND PROTEIN CONCENTRATION OF SEPHADEX G200 FRACTIONS OF POOLED P-K POSITIVE RSD SERUM AFTER CONCENTRATION AND DIALYSIS.

FRACTION	VOLUME (ml)	CONC.(g/L)
1	0.5	3.5
2	0.8	11.7
3	0.8	11.9
4	1.0	21.7
5	0.7	10.0
6	0.5	11.5
7	0.5	2.4
8	0.5	n.d.

n.d. = not detectable.

apparent at 6 hours which became marked by 24 hours and was still present, though diminished, at 48 hours. Initially the reactions were isolated at each site but gradually became confluent to involve the entire test area. The recipient animal violently resented handling of the test site throughout this period. A similar reaction was observed after I/D challenge of a second recipient with the same fractions.

In both recipients a slight response to the PBS controls was apparent for up to 2 hours after inoculation,

However, PBS passed through the column and filtered under the conditions described, resulted in marked reactions after I/D injection into both recipients and a third experimental horse.

A second column was prepared under the same conditions as the first using fresh Sephadex G200. Pooled, concentrated and dialysed fractions of P-K positive R.S.D. serum from this column resulted in similar untoward reactions to those previously described when inoculated I/D into an experimental horse.

These adverse reactions in horses to I/D inoculation of Sephadex G200 eluate prompted an investigation into the nature and cause of the reaction as such adverse dermal reactions have not been reported in other species.

11.4.2. INVESTIGATION OF ADVERSE REACTIONS IN HORSE SKIN FOLLOWING I/D INOCULATION OF SEPHADEX G200 ELUATE

11.4.2.(i) Bacteriology

Cultural examinations of both the G200 column slurry and the membrane filtered eluate failed to demonstrate the presence of bacteriological contamination.

(ii) Examination of the gel swelling effluent

10g of recently purchased Sephadex G200[1] was allowed to swell in excess PBS for 48 hours and 3mls of the supernatant fluid was decanted. Following repeated passage through a 0.22μ membrane filter, 0.1ml was injected I/D into a previously unchallenged horse

1 Pharmacia Fine Chemicals, Uppsala, Sweden.

along with 0.1ml PBS as a control. Both sites were biopsied at 6 hours using a 5mm punch biopsy after local anaesthetic infiltration. Following fixation in 10% formalin, paraffin block sections were routinely prepared and stained with haematoxylin and eosin.

The horizontal diameters (mm) of the reactions at both sites at 1, 2, 4, 6 and 24 hours after inoculation are shown in Table 11.4a and the results of the 6 hour biopsies presented in Table 11.4b.

TABLE 11.4a

HORIZONTAL DIAMETER (mm) OF THE REACTIONS RESULTING FROM I/D INOCULATION OF PBS-SEPHADEX G200 SLURRY EFFLUENT.

TIME (HOURS)	PBS-SEPHADEX G200 SLURRY EFFLUENT	PBS CONTROL
1	14	12
2	17	13
4	25	D
6	27	D
24	D*	D

D - Dispersed

D* - Diffuse, raised painful lesion.

TABLE 11.4b

HISTOPATHOLOGY OF A 6 HOUR BIOPSY FROM BOTH SITES RECORDED IN TABLE 11.4a.

DERMAL CHALLENGE	BIOPSY
PBS Control	Normal epidermis, no evidence of inflammatory reaction or oedema in dermis.
PBS - G200 slurry effluent	Normal epidermis. Moderate perivascular infiltration of neutrophil polymorphs in dermis, with neutrophil migration from blood vessels. These signs indicate an early exudative inflammatory reaction in the dermis.

(iii) Ultracentrifugation of the Sephadex G200 column eluate

36ml of PBS eluate from the Sephadex G200 column was collected during washing of the column under conditions identical to those used during serum fractionation (11.4.1). Following concentration by evaporation to 10ml and dialysis against repeated changes

of PBS for 24 hours, the eluate was passed through a 0.22μ membrane filter and 5ml of the eluate was centrifuged at 50,000rpm for 30 minutes in a Beckman L265B centrifuge[1] using a Beckman rotor type SW65K. After removal of the top 4.5ml supernatant the remainder was resuspended in 4.5ml fresh PBS. The absorbance at 280nm of the precentrifuged eluate, the supernatant and the resuspended 'deposit' was determined against a distilled water control using a Cecil CE292 Ultraviolet spectrophotometer[2], and 0.1ml of each was injected I/D into an experimental horse. The resulting reactions along with that of a PBS control were measured at 1, 2, 4, 6 and 24 hours.

The results are presented in Tables 11.5a and 11.5b.

TABLE 11.5a

U/V ABSORBANCE OF PRE- AND POST CENTRIFUGATION SEPHADEX G200 COLUMN ELUATE PREPARATIONS.

Eluate Preparation	Absorbance at 280 nm.
Distilled water control	0.000
PBS Control	0.002
Precentrifuge PBS eluate	0.096
Post centrifugation supernatant	0.087
Post centrifugation resuspended 'deposit'	0.011

1. Beckman Scientific Instruments, High Wycombe, England.
2. Cecil Instruments, Cambridge, England.

TABLE 11.5b

HORIZONTAL DIAMETER (mm) OF REACTIONS RESULTING FROM I/D INOCULATION OF THE PRE- AND POST CENTRIFUGATION SEPHADEX G200 ELUATE PREPARATIONS.

CHALLENGE	TIME (HOURS)				
	1	2	4	6	24
PBS	10	12	D	D	D
Precentrifuge column eluate	11	11	17	20	D*
Post centrifuge supernatant	11	12	15	15	D*
Post centrifuge resuspended 'deposit'	11	14	19	23	D*

D - Dispersed

D* - Diffuse, raised painful lesion.

The results of I/D challenge shown in Table 11.5b indicate that the causal agent of the adverse dermal reaction is an insoluble or macromolecular moeity sedimented in the ultracentrifugal 'deposit'. However, the response to challenge with the ultracentrifugal supernatant suggests the possible involvement of a second soluble agent, or variation in molecular size of a single polymeric agent. The U/V absorbance of the PBS column eluate probably results from elution of protein trapped in the column during previous serum fractionation. However, the low U/V

absorbance of the resuspended deposit in comparison with the supernatant indicates a relatively lower concentration of aromatic or heterocyclic residues in the deposit (Van Holde, 1971) and suggests that the causal agent/s is not a polypeptide or protein.

(iv) Investigation of the presence of circulating precipitating antibodies against the resuspended deposit after ultracentrifugation of Sephadex G200 - PBS eluate.

Sephadex G200 gel beads are prepared from cross linked dextrans. High molecular weight dextrans can induce small amounts of circulating antibody in man (Kabat and Berg, 1953; Allen and Kabat, 1958), although circulating anti-dextran precipitating and haemagglutinating antibodies have been demonstrated in individuals with no history of dextran administration (Kabat and Berg 1953; Hedin, Richter and Ring, 1976). The occurrence of a primarily perivascular reaction in the 6 hour biopsies following I/D challenge with Sephadex G200 effluent suggested the possibility of anti-dextran precipitins in the horse resulting in an Arthus-like response after I/D antigen challenge. The sera of a number of horses was examined by immunodiffusion for the presence of anti-Sephadex antibodies.

A double immunodiffusion plate technique was used in which 20mls of 1% agar[1] in 0.85% NaCl, 0.1M Tris-HCl (pH 7.6) and 0.2M Tris-barbital high

1 Ionagar; Oxoid Ltd., Basingstoke, Hants.

resolution buffer (pH 8.8)[1] were poured into sterile plastic petri dishes. A single central and six symmetrically placed peripheral wells were cut in the gel. O.1ml of the resuspended ultracentrifuge 'deposit' (11.4.2.iii) was applied to the central well and O.1ml each of 6 equine sera, 2 of which were from experimental horses previously shown to react to I/D G200 eluate challenge, were applied to the peripheral wells. The plates were incubated in a humidity chamber at 37°C and examined at 24 hour intervals for 7 days.

No immunoprecipitation was observed on any of the plates during 7 days incubation.

(v) Dermal reactions to sephadex G200 eluate: Discussion

The results of this investigation suggest that the causal agents of the untoward dermal responses are probably dextran aggregates, which on the basis of their size (< 0.22μm) may have resulted from mechanical disruption of the 40-120μm diameter Sephadex G200 gel beads during preparation and running of the column. However, the gel slurry and the column were prepared according to the manufacturer's instructions, with prolonged washing of the packed column with PBS prior to use to remove 'fines'. In addition, the buffer flow rate in this series, 27ml/hour, was within the 30ml/hour calculated maximum recommended flow rate for a Sephadex G200 column of these dimensions where a

1 Gelman, Michigan, U.S.A.

peristatic pump is also used, and is comparable to the flow rates in relation to the column dimensions used by other authors examining the elution pattern of P-K activity in mammalian sera.

The post-elution concentration, dialysis and membrane filtering procedures in this series are not markedly different from those of Reid et al., (1968) and Barratt (1973), except that these authors used a negative pressure or osmotic system of fraction concentration.

Endogenous enzymatic degradation of the gel structure may have arisen following microbial contamination of the column bed, although in this case no such contamination was found.

The failure to demonstrate detectable levels of precipitating antibody against Sephadex effluent in the six sera examined does not totally exclude the possibility of an immunologically mediated dermal response to Sephadex in the challenged horses. Hedin et al., (1976) demonstrated reactions for up to several hours after I/D dextran challenge of human subjects with high titres of dextran reactive antibodies (DRA), which they interpreted as being caused by the activation of vasoactive mediators by immune complexes. However, these same authors also demonstrated skin responses in individuals with no haemagglutinating or PCA mediating DRAs and, noting that certain macromolecules can liberate cellular vasoactive compounds independently of an immune

mechanism, suggested that these dermal responses may arise from the direct release of vasoactive mediators by inoculated dextran.

In spontaneously dextran reactive rats, parenteral and I/D administration of dextrans can result in systemic and cutaneous anaphylactic-like reactions respectively (Vorhees, Baker and Pulaski, 1951; Beraldo, Dias da Silva and Lemos Fernandes, 1961), and can induce histamine release from isolated peritoneal mast cells in the absence of homologous serum (Baxter 1972). However, Baxter and Adamik (1976) failed to demonstrate the presence of passively transferable or PCA mediating anti-dextran antibodies in the serum of spontaneously dextran reactive rats and demonstrated the failure of mast cell homogenate from such rats to passively sensitize the skin of non-dextran reactive rats, indicating the presence of natural receptors for dextran on the mast cells of dextran reactive rats.

Although spontaneously occurring dextran reactive antibodies or dextran sensitivity in the horse has not been investigated, the ability of horses to experimentally produce anti-pneumococcal polysaccaride antibodies is well established (Heidelberger and Pedersen, 1937; Kabat, 1961). Zolla and Goodman (1968) and Helms and Allen (1970) have shown that the major antibody in antipneumococcal horse serum is a salt dissociable, noncovalently linked aggregate of a 6.3S globulin which is antigenically distinct from

IgG, IgG(T) and IgM. This aggregating immunoglobulin (AI) or 'gamma-1 component' appears in the serum of normal horses at levels of around 39mg/100ml and precipitates pneumococcal polysaccaride and fixes complement (McGuire, Crawford and Henson, 1972). Although the antigenic inter-relationship between naturally occurring AI and the various known pneumococcal polysaccaride antigen types has not been fully elucidated, the antigenic cross reactivity of synthetic dextrans and certain pneumococcal polysaccaride antigens has been established (Hehre, Sugg and Neill, 1952), suggesting that circulating AI could mediate a dermal response following Sephadex dextran challenge in horses. However such circulating precipitating antibodies were not detected using the double immunodiffusion method in the present series, although the sensitivity of this method may not permit their detection. Intravenous dextrans have been used therapeutically in horses by Greatorex (1975), who reported muscular fasiculations, tachycardia and collapse in 8 out of 55 treated animals. These reactions resembled those of passive systemic anaphylaxis (Ritzenthaler, 1924), suggesting a mast cell based mechanism may be involved.

The apparent sensitivity of horse skin to dextran aggregates or 'fines' occurring in the Sephadex G200 eluate precludes the use of this medium in the fractionation of reaginic serum for subsequent I/D testing in

the horse. However Halliwell (1979, pers. comm.) encountered no adverse reactions following I/D challenge with Sephadex G200 eluate, although Quinn and Baker (1979, pers. comm.) did encounter marked, non specific responses following I/D challenge of experimental horses with Culcoides antigen fractions eluted during sucrose density gradient ultracentrifugation. The contradictory findings of Halliwell with those in this study are probably due to differences in the chromatography techniques.

11.4.3. ION EXCHANGE CHROMATOGRAPHY

(i) Introduction

Ion exchange chromatography has been widely used in the study of reaginic antibodies to examine the elution characteristics of the P-K mediating antibody in relation to known protein components, (Humphrey and Porter, 1957) and to isolate the reagin (Ishizaka and Ishizaka, 1967). The principles of ion exchange chromatography have been described by Fahey and Terry (1973). This technique utilises the electrostatic binding of proteins to a buffered cellulose resin suspension and the subsequent differential elution of the bound proteins according to their molecular charge by varying the buffer pH or molarity.

(ii) Materials and Methods

DEAE anion exchange cellulose[1] was prepared as follows. After precycling the cellulose and

1 Whatman Ltd., Maidstone, Kent.

subsequent removal of 'fines' according to the manufacturer's instructions, the defined degassed DEAE-cellulose was poured into a 25 x 1.5cm column and equilibrated at room temperature with the starting buffer 0.04M Tris-HCl (pH 8.0), the stabilized cellulose having a final bed height of 17cms. After a series of trials using normal horse serum, 3 ml of pooled P-K positive R.S.D. was dialysed for 6 hours against starting buffer and applied to the column. A linear buffer gradient, increasing from 0.04M Tris-HCl (pH 8.0) to 0.5M Tris-HCl (pH 8.0), was applied to the column with an LKB Ultrograd 11300[1] over 16 hours, using a peristaltic pump with a constant flow rate of 40ml per hour. Approximately 7ml fractions were collected at 10 minute intervals. Collection and monitoring of the eluate was carried out as previously described (7.2.2.).

The collected fractions were pooled according to the protein distribution into 12 distinct peaks (Fig. 11.1) and concentrated by evaporation to 0.5ml. After dialysis against PBS for 48 hours at 4°C, followed by dialysis against sterile physiological saline for a further 24 hours, the final fractions were passed through a 0.22μ membrane filter and stored in sterile plastic bijoux bottles at -20°C until required.

P-K testing of the individual pooled fractions was carried out as previously described (10.2) and included

1 LKB Instruments, Bromma, Sweden.

prefractionation R.S.D. serum as a positive sensitization control.

Immunoelectrophoresis of the pooled fractions against rabbit anti-whole horse serum[1] was carried out using the slide method of Hirschfeld (1960) employing 1% agar[2] and a continuous barbital-calcium lactate buffer system (pH 8.6) in the electrophoretic separation phase. Control wells contained a mixture of normal horse serum and 2% bromophenol blue dye as an albumin marker. The immunoelectrophoresis patterns were interpreted according to the data of Allen and Dalton (1975).

The presence of IgG, IgG(T) and IgM in the final protein fractions was determined using a slide double immunodiffusion technique employing 1% agar[2] in 0.1M Tris-HCl (pH 8.0). Approximately 30µl of each pooled fraction was applied to a central well and 30µl of commercially available antisera was applied to 3 symmetrically placed peripheral wells. The following Fc specific antisera were used; rabbit anti-horse IgG,[1] rabbit anti-horse IgM[1] and goat anti-horse IgG(T)[1]. The slides were incubated in a humidity chamber for 48 hours at 37°C.

(iii) Results

The pattern of protein recovery from DEAE-cellulose column in relation to the linear increase in buffer molarity is shown in Figure 11.1. along with

1 Miles Laboratories, Slough, England.
2 Ionagar; Oxoid Ltd., Basingstoke, Hants.

the distribution of the individual protein peaks 1-12.

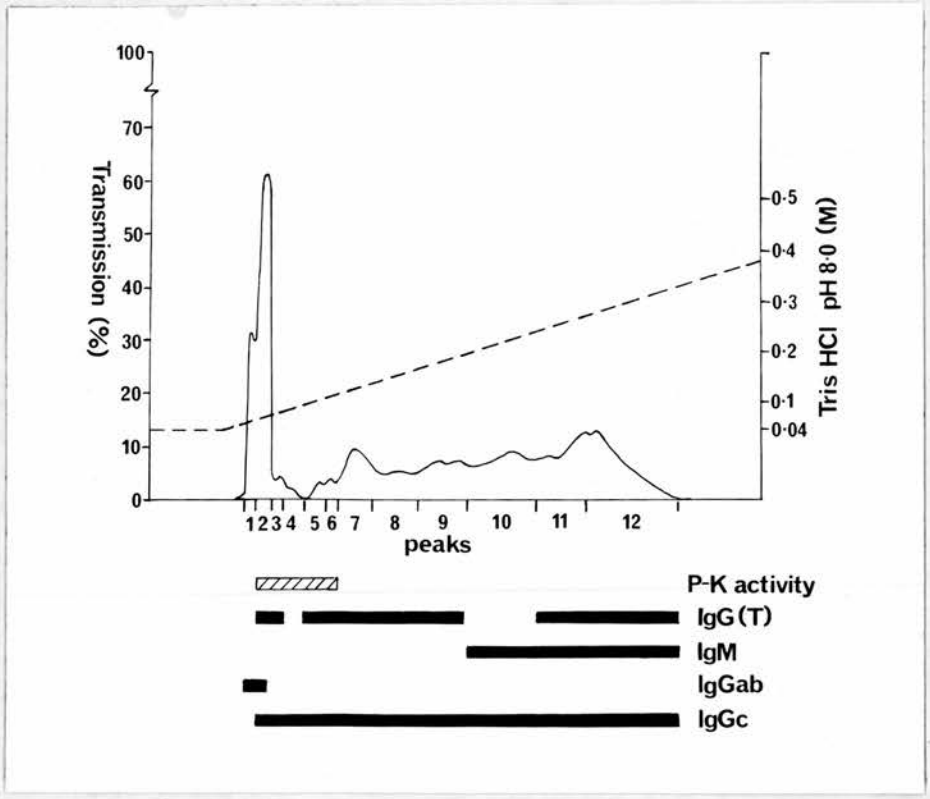


Fig. 11.1.

Elution pattern of horse serum proteins (peaks 1-12) on DEAE 52 chromatography using a linear Tris-HCl (pH 8.0) gradient, showing the relative distribution of P-K activity and the immunoglobulin classes Gab, Gc, M and G(T).

The distribution of detectable P-K activity in peaks 1-12 is shown in Table 11.6. This activity was eluted in peaks 2-6, over a range of Tris-HCl buffer molarity of 0.05M - 0.14M (Fig. 11.1). However, unlike the biphasic P-K response elicited after sensitization with whole serum, the responses elicited at sites sensitized

with DEAE peaks 2-6 were monophasic, reaching a maximum around 60 minutes, and in the case of peaks 4 and 5 were inapparent at 360 minutes.

TABLE 11.6

THE DISTRIBUTION OF P-K ACTIVITY IN THE ELUATE FOLLOWING DEAE 52 CHROMATOGRAPHY OF POOLED P-K POSITIVE R.S.D. SERUM: THE HORIZONTAL DIAMETER (mm) OF THE REACTION RESULTING FROM ANTIGEN CHALLENGE OF HOMOLOGOUS DERMAL SITES SENSITIZED WITH PROTEIN PEAKS 1-12.

PEAKS	TIME (MINUTES)						P-K ACTIVITY
	0	30	60	120	360	24 HOURS	
1	11	12	12	11	D	D	
2	10	17	15	15	16	D	+
3	11	14	17	15	12	D	+
4	11	18	16	14	D	D	+
5	12	19	21	20	D	D	+
6	11	16	16	16	16	D	+
7	10	10	10	9	9	D	
8	9	10	12	13	D	D	
9	11	13	12	12	12	D	
10	10	11	13	11	D	D	
11	11	11	12	11	D	D	
12	10	9	12	14	13	D	
Foal serum sensitization control	10	11	11	12	D	D	
Positive serum sensitization control	11	17	17	20	23	D	+
Antigen challenge control	9	9	10	D	D	D	

D = Dispersed.

Immunoelectrophoresis of peaks 1-12 against anti-whole horse serum is shown in Figure 11.2, and slide

double immunodiffusion of peaks 1-12 against anti-horse immunoglobulin sera is shown in Figure 11.3. The distribution of the P-K activity in relation to the distribution of these immunoglobulin classes after DEAE chromatography is illustrated in Figure 11.1. The IgGab and IgGc subclasses were differentiated by immunoelectrophoresis (Fig. 11.2), IgGab forming the bifid, most cathodal arc in the case of peaks 1 and 2 (Allen and Dalton, 1975). With the exception of peaks 11 and 12 the immunoglobulin distribution determined by slide double immunodiffusion and by immunoelectrophoresis was identical. In the case of peaks 11 and 12, all three immunoglobulin classes were demonstrable by immunodiffusion against specific antisera though not by immunoelectrophoresis against anti-whole horse serum. The IgG in peaks 11 and 12 probably is of the IgGc subclass.

A double precipitin arc was apparent around the anti-IgG well after double immunodiffusion of peaks 6-12 (Figure 11.3) probably resulting from contamination of the antiserum with antibodies against a serum protein eluted from DEAE-cellulose over the Tris-HCl molar range 0.14M-0.33M. This contaminant is not IgG(T) or IgM, although its relative distribution after DEAE-cellulose chromatography suggests that it may be IgA (Vaerman et al., 1971). Such contamination is a hazard of using commercially produced antisera.

Fig. 11.2. Immunoelectrophoresis of whole horse serum and DEAE-cellulose chromatography peaks 1-12 of pooled P-K positive R.S.D. serum against rabbit anti-whole horse serum.

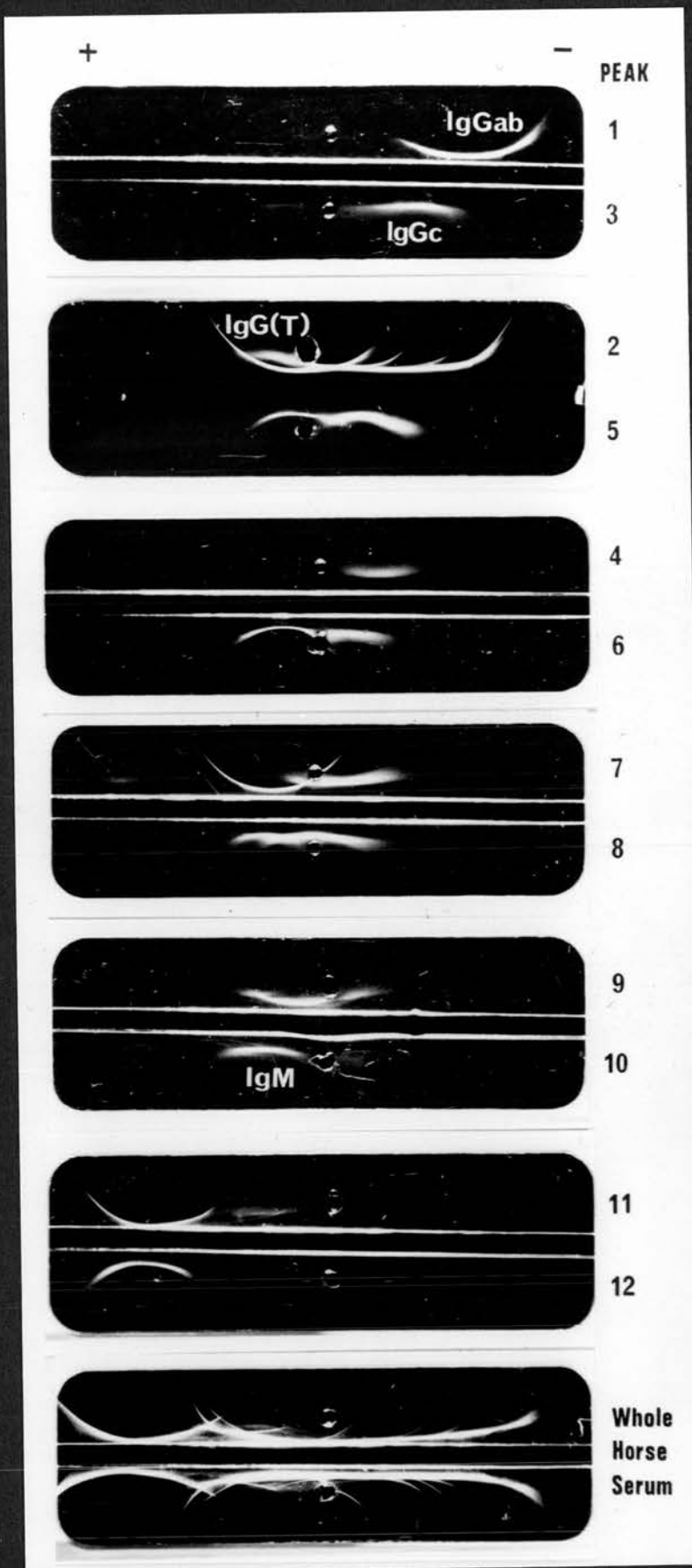
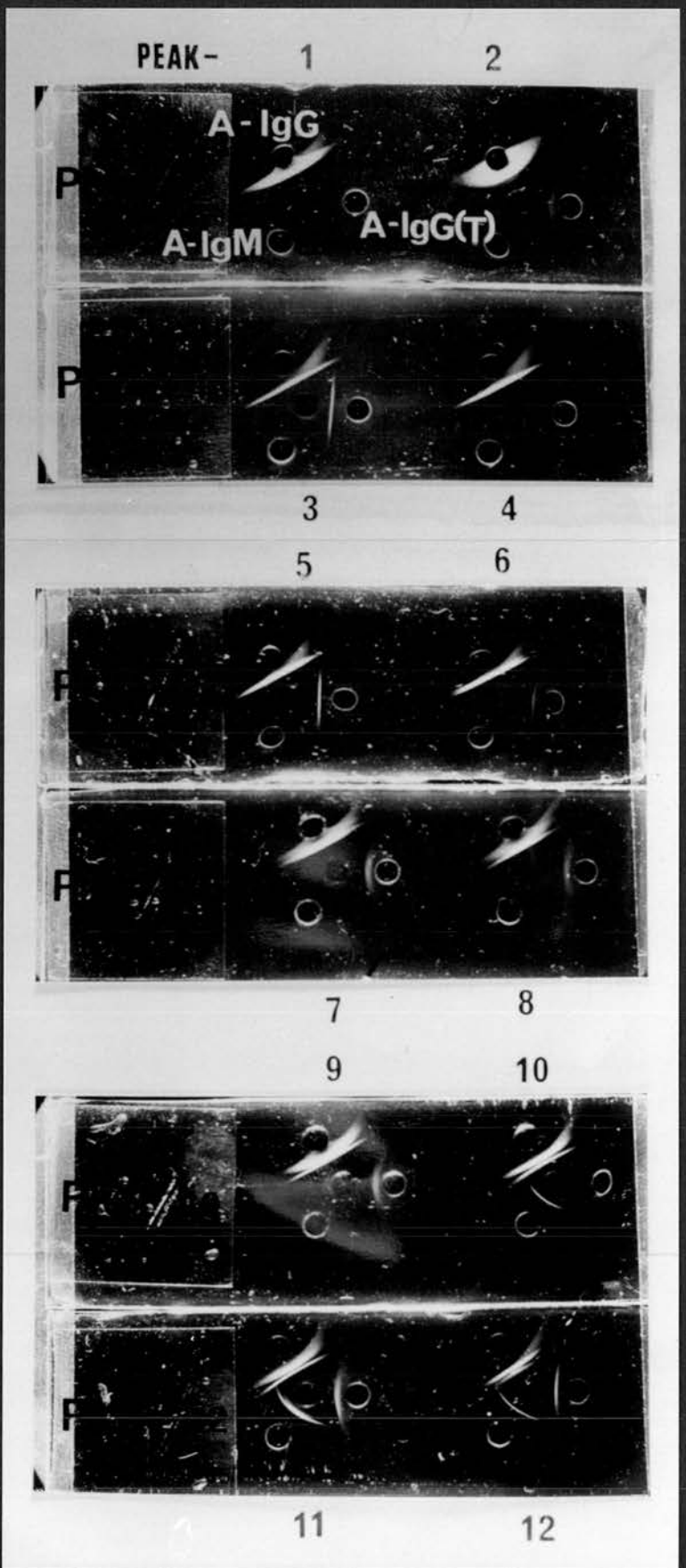

+
−
PEAK
IgGab
1
3
IgGc
IgG(T)
2
5
4
6
7
8
9
IgM
10
11
12
Whole
Horse
Serum

Fig. 11.3. Double immunodiffusion of DEAE-cellulose peaks 1-12 of pooled P-K positive R.S.D. serum against anti-horse IgG, IgM and IgG(T) sera.


PEAK-
1
2
A-IgG
A-IgM
A-IgG(T)
3
4
5
6
7
8
9
10
11
12

(iv) Discussion

The relative distribution of the immunoglobulin classes IgG, IgG(T) and IgM after DEAE-cellulose chromatography of horse serum using a Tris-HCl (pH 8.0) buffer gradient (Fig. 11.1) is similar to that demonstrated by Vaerman et al., (1971) using a stepwise Tris-NaCl (pH 8.0) gradient. The distribution of the IgG and IgM classes is similar to that of human IgG and IgM after DEAE-Sephadex chromatography of whole human serum using a Tris-HCl (pH 8.0) buffer gradient (Johansson et al., 1968; Perelmutter and Liakopoulou, 1971). The appearance of precipitin arcs against all three horse immunoglobulin classes after immunodiffusion, but not immunoelectrophoresis, of peaks 11 and 12 may reflect the greater sensitivity of the former technique which utilises monospecific antisera.

P-K activity in the DEAE-cellulose eluate was detected overlapping in part the major IgG peak and extending into the trailing IgG fraction, but not overlapping the IgM fraction. After DEAE-Sephadex chromatography of whole reaginic human serum or isolated immunoglobulin fractions using Tris-HCl (pH 8.0) buffer the P-K activity is similarly distributed about the IgG and IgM peaks and is eluted in the molar range 0.1M-0.3M (Ishizaka and Ishizaka, 1967; Johansson et al., 1968; Perelmutter and Liakopoulou, 1971). Although the Sephadex chromatography medium used by these

authors differs from the cellulose medium used in the present study, the DEAE anion exchange group is the same and the similarity in elution characteristics of the homocytotropic antibody in horse and human serum, particularly in relation to the major immunoglobulin classes, indicates some similarity in net molecular charge. Furthermore, after DEAE-cellulose chromatography of dog serum, using a phosphate buffer (pH 8.0), the homocytotropic antibody is eluted after the first IgG peak and in the trailing IgG fraction, (Halliwell, et al., 1975) suggesting some similarity in net charge of the homocytotropic antibody in horse and dog serum.

The distribution of detectable P-K activity after DEAE chromatography of horse serum does not parallel that of the immunoglobulin classes G, G(T) and M, suggesting that the activity resides outwith these classes. However the possibility remains that the equine homocytotropic antibody activity may be associated with the remaining immunoglobulin classes; IgA, AI (aggregating immunoglobulin) and $1OS_{\gamma 1}$ immunoglobulin. The respective biological roles of AI and the $1OS_{\gamma 1}$ immunoglobulin have not been elucidated. However the latter immunoglobulin, occurring in anti-β lactoside sera, is sensitive to 2-mercaptoethanol reduction (Raynaud and Iscaki, 1970) and probably merits further investigation of it's relationship with the homocytotropic antibody.

The results of this experiment show that the P-K responses elicited following antigen challenge of sites sensitized with the DEAE-cellulose chromatography fractions differs morphologically from the response elicited at sites sensitized with whole R.S.D. serum, the former being an immediate monophasic response in contrast to the latter biphasic response. In addition, isolated delayed responses were not observed at sites sensitized with any of the DEAE-cellulose fractions. This observation suggests that although the immediate response is wholly homocytotropic antibody dependent, the delayed response, while dependent upon a preceding or initiating immediate response, involves one or more unidentified additional factors. However, the involvement of IgG immune complexes as mediators of the delayed response may be excluded because of the occurrence of IgG in the P-K active fractions. Solley et al., (1976) failed to demonstrate the deposition of IgG immune complexes in directly induced late cutaneous allergic responses in man, although IgM immune complex deposition was observed in 2 out of 15 cases examined, and C_3 deposition was observed in one case.

A second explanation of the absence of delayed P-K responses at sites sensitized with DEAE-cellulose fractions of R.S.D. serum is that should the delayed response be dependant upon high sensitizing titres of homocytotropic antibody then dilution and loss of

the antibody during fractionation may have resulted in insufficient titres in the final fractions to initiate a delayed response. It has been shown that large amounts of human homocytotropic antibody are irreversibly lost during DEAE chromatography (Stanworth, 1963) and concentration and dialysis (Stanworth, 1959; Bazarat and Hamburger, 1972).

CHAPTER 12

EVIDENCE OF ANTIGENIC CROSS REACTIVITY BETWEEN EQUINE HOMOCYTOTROPIC ANTIBODY AND HUMAN IMMUNOGLOBULIN E.

12.1. INTRODUCTION

Antigenic cross reactivity between human IgE and reagin-like antibodies in the monkey, pig, dog and ox has been reported (Table 9.1a), and more recently a similarly cross reactive protein has been reported in the serum of a marsupial, the quokka (Lynch and Turner, 1974). However, antigenic cross reactivity of human and mouse reagin cannot be demonstrated (Schwartz and Levine, 1973), and the evidence of human IgE cross reactivity with guinea pig and rabbit reagin is contradictory (Dobson, Rockey and Soulsby, 1971; Levine, Change and Nelson, 1971; Zvaifler and Robinson, 1969; Ishizaka, Ishizaka and Hornbrook, 1970).

These heterologous reagin-anti human IgE interactions have been demonstrated by a number of techniques, including immunoabsorption in the monkey (Ishizaka and Ishizaka, 1968), pig (Barratt, 1972), calf (Doyle, 1973) and dog (Halliwell *et al.*, 1972), immunodiffusion in the monkey (Ishizaka, Ishizaka and Tada, 1969), immunofluorescence in the dog (Healey and Gaafar, 1977), and also by reversed cutaneous anaphylaxis in the dog (Halliwell *et al.*,1972), quokka (Lynch and Turner, 1974) and monkey (Ishizaka and Ishizaka, 1968).

In this chapter the ability of anti-human IgE to cross react with horse homocytotropic antibody was investigated in accordance with Vaerman's first criterion of interspecies protein homology. The techniques used were; the induction reversed cutaneous anaphylaxis, immunoabsorption and immunofluorescence.

12.2. ANTI-HUMAN IgE INDUCED REVERSED CUTANEOUS ANAPHALAXIS-LIKE RESPONSES IN THE HORSE.

12.2.1. INTRODUCTION

Ishizaka and Ishizaka (1968) described the induction of typical wheal and flare responses following I/D injection of anti-human IgE into human volunteers. This reaction was described as reversed cutaneous anaphylaxis (RCA) and I/D injection of anti-human IgG, IgM, IgA and IgD serum failed to induce similar responses. Subsequently Ishizaka and Ishizaka (1969) demonstrated that the apparent bridging of adjacent IgE molecules on the surface membranes of basophils by the bivalent $F(ab^1)_2$ fragment of an antibody directed specifically against the Fc antigenic determinants on the IgE molecules resulted in the release of intracellular mediators, and it was postulated that a similar mechanism probably resulted in the anti-IgE mediated RCA responses via IgE coated dermal mast cells.

Thus, in the event of common heavy chain antigenic determinants existing on the Fc fragments of heterologous reaginic antibodies, inoculation of Fc specific anti-human IgE sera will potentially elicit RCA-type responses in heterologous skin. Yet, only in the dog and quokka has this relatively simple indicator of shared antigenic determinants been described (Halliwell et al., 1972; Lynch and Turner 1974).

In the present series of experiments the induction of RCA-like responses in horse skin by anti-human IgE is studied and compared to the responses elicited by inoculation of antisera against various horse immunoglobulins and immunoglobulin fragments.

12.2.2. MATERIALS AND METHODS

(i) Horses

8 horses from the experimental stock previously described were used (10.2.2.).

(ii) Intradermal Procedures

Lateral cervical test sites were used throughout.

(a) Anti-human IgE: 0.1ml Fc specific sheep anti-human IgE serum (Sh Ah IgE)[1] and fresh sheep serum were injected I/D into horses 1 to 6. The response to anti-globulin and control serum challenge was recorded as the horizontal diameter of the reaction (mm) at 0, 30, 60, 120 and 360 minutes, and at 24 hours.

(b) Anti-horse immunoglobulins: 0.1 ml of the anti-immunoglobulin serum and the respective control serum was injected I/D into the experimental horses as follows: Fc specific rabbit anti-horse IgG serum (Rb Aeq IgG)[2] into horses 6, 7 and 8. Rabbit anti-whole horse serum (Rb Aeq WS)[2] into horses 1 and 2. Fc specific goat anti-horse IgG(T) serum (Gt Aeq IgG(T)[2]) into horse 1, and pig anti-horse light chain (kappa and lamda determinants) serum (Sw Aeq Lc)[2] into horse 3.

1. Nordic Immunological Laboratories, Maidenhead, England.
2. Miles Laboratories Ltd., Slough, England.

The antibody activity of the test antisera was checked by immunoelectrophoresis prior to I/D use.

The dermal responses were recorded as in (a).

(iii) Dermal Biopsy

Sh Ah IgE and control sheep serum were each inoculated at 3 sites in horses 4 and 5. Rb Aeq IgG and control rabbit serum were each inoculated at 3 sites in horses 7 and 8. The three test and control sites on each animal were biopsied at 30 minutes, 4 hours and 24 hours respectively. Full thickness dermal biopsies were removed from the centre of the lesion using an 8mm biopsy punch following local anaesthesia with 2% lignocaine. The biopsy specimens were transferred immediately into Bouin's fixative and after processing to wax, 6μ sections were prepared and stained with Haematoxylin and Eosin, Geimsa, Carbol Chromotrope and Toluidine Blue (pH 4.2) using standard histochemical methods.

12.2.3. RESULTS

(i) Intradermal Procedures

The horizontal diameters of the reactions at increasing intervals after I/D inoculation of Sh Ah IgE and the various anti-homologous whole serum and immunoglobulin sera are presented in Table 12.1. The reactions elicited in horse 6 by I/D Sh Ah IgE and Rb Aeq IgG challenge are shown in Figure 12.1. The changes in mean πr^2 (cm^2) of the reactions elicited by

TABLE 12.1.

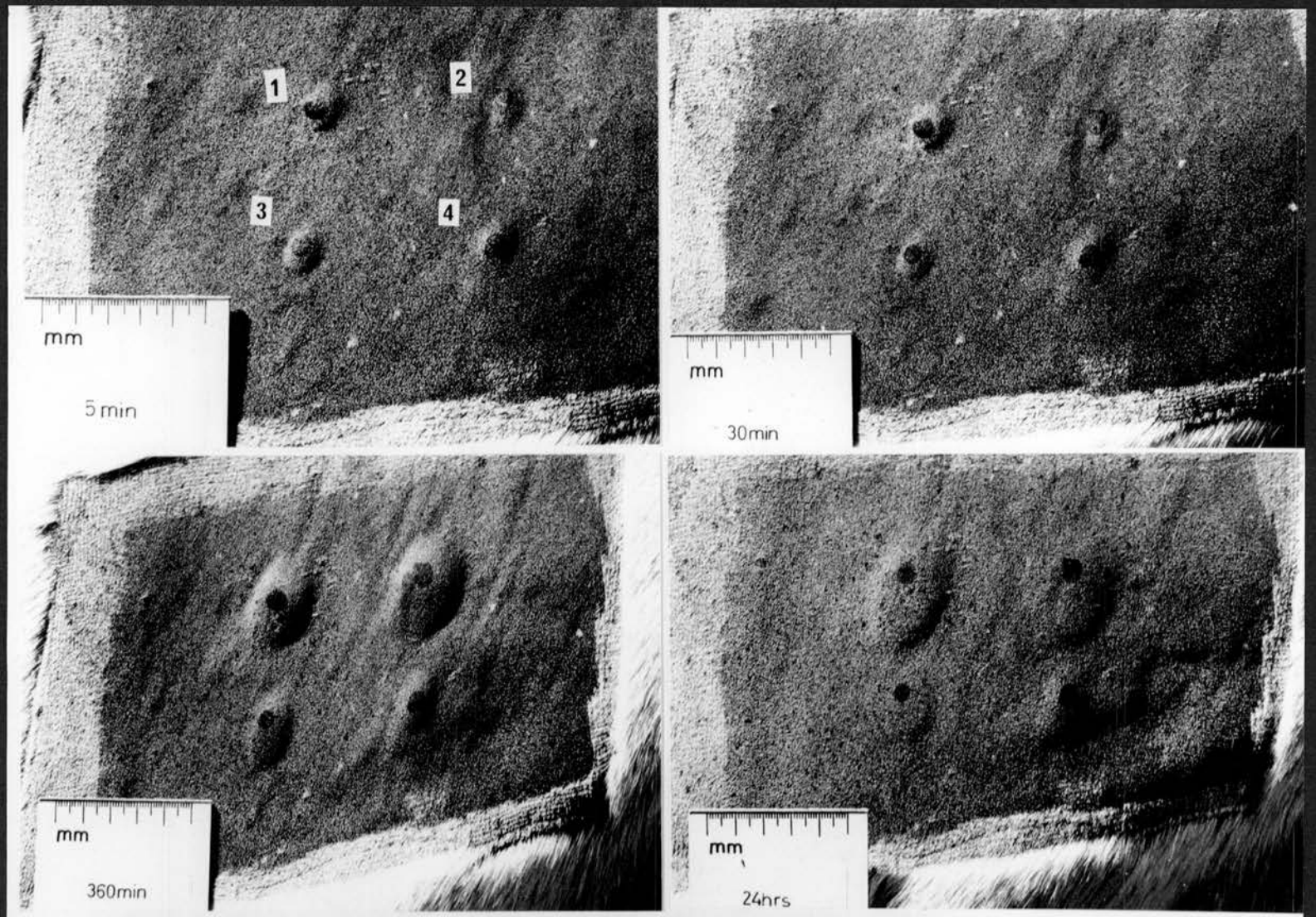
THE RESPONSE OF NORMAL HORSE SKIN TO INTRADERMAL CHALLENGE WITH ANTI-HUMAN IgE, ANTI-WHOLE HORSE SERUM, AND ANTI-HORSE IgG, IgG(T) AND IMMUNOGLOBULIN LIGHT CHAINS: EXPRESSED AS HORIZONTAL DIAMETER (mm) OF THE REACTION.

CHALLENGE	Horse Number	MINUTES 0	30	60	120	360	24 HOURS	NOTE
Sheep anti-human IgE	1	12	18	20	22	28	D	
	2	12	19	20	22	27	D	
	3	10	16	21	23	24	D	
	4	10	17	17	22	22	D	BIOPSIED
	5	10	16	20	22	24	D	BIOPSIED
	6	10	16	17	19	26	DIFFUSE RAISED AREA	PHOTOGRAPHED
Sheep Serum	1	10	11	D	D	D	D	
	2	10	10	D	D	D	D	
	3	10	10	12	12	D	D	
	4	10	10	10	D	D	D	BIOPSIED
	5	10	12	13	13	D	D	BIOPSIED
	6	12	12	12	13	17	DIFFUSE RAISED AREA	PHOTOGRAPHED
Rabbit anti-horse IgG	6	10	10	13	17	29	DIFFUSE RAISED AREA	PHOTOGRAPHED
	7	6	6	10	15	21	DIFFUSE RAISED AREA	BIOPSIED
	8	10	8	10	13	20	D	BIOPSIED
Rabbit Serum	6	13	14	14	14	13	DIFFUSE RAISED AREA	PHOTOGRAPHED
	7	6	6	8	7	D	D	BIOPSIED
	8	10	8	10	8	12	D	BIOPSIED
Rabbit anti-whole horse serum	1	10	14	14	22	50	55*	
	2	10	13	14	25	50	D	
Rabbit Serum	1	10	11	11	10	D	D	
	2	10	13	13	D	D	D	
Pig anti-horse light chains	3	10	14	14	25	25	D	
Pig Serum	3	10	14	13	6	D	D	
Goat anti-horse IgG(T)	1	10	12	13	15	20	D	
Goat Serum	1	10	11	14	16	20	D	

D - Dispersed.

* - Reaction present at 48 hours as a discrete raised plaque.

Fig. 12.1. Response of horse skin to I/D challenge with sheep anti-human IgE, rabbit anti-horse IgG and their respective serum controls.



1- SHEEP ANTI-HUMAN IgE. 2- RABBIT ANTI-HORSE IgG. 3-SHEEP SERUM. 4- RABBIT SERUM.

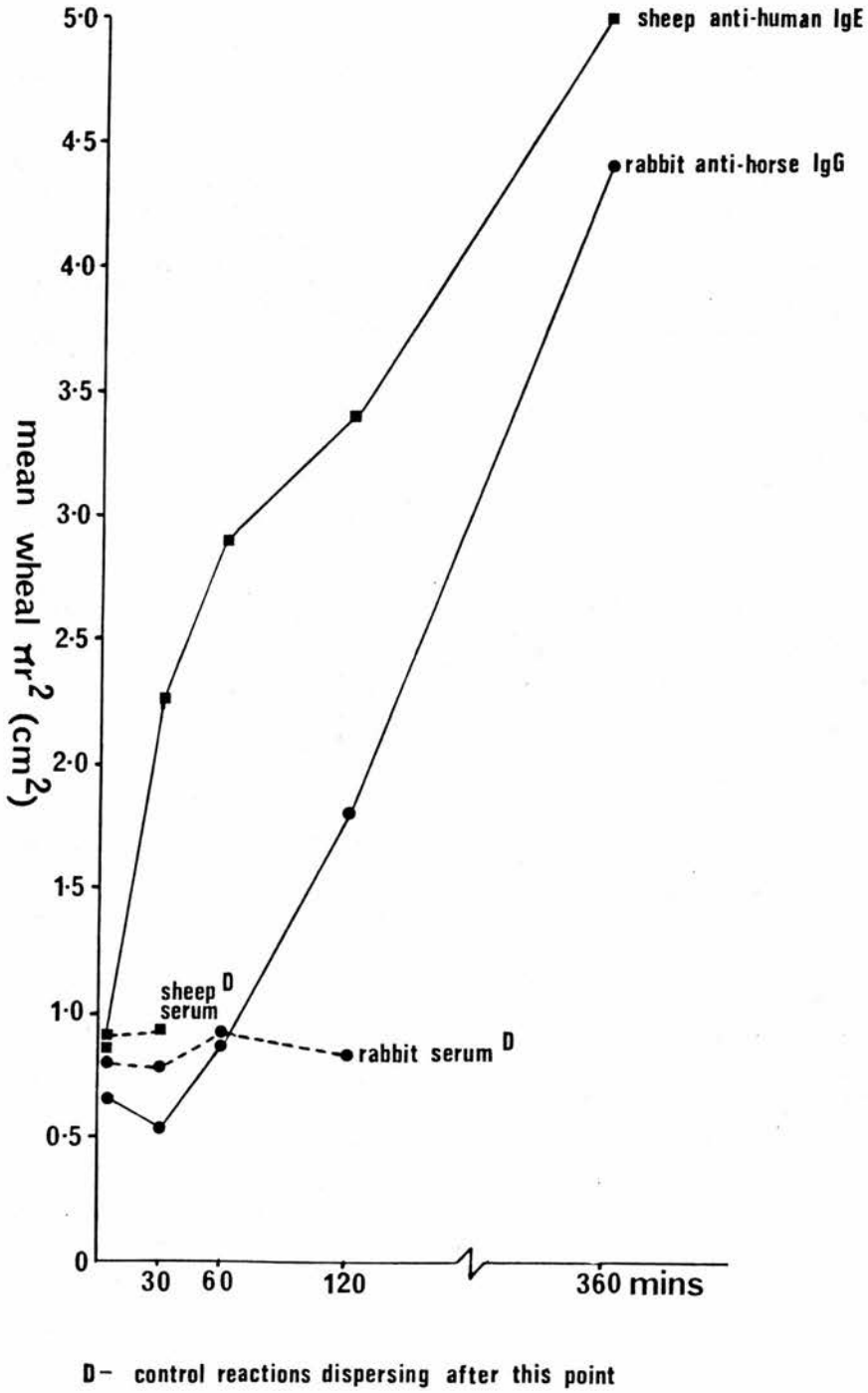


Fig. 12.2. Mean πr^2 of the reactions in horse skin after I/D inoculation of sheep anti-human IgE, rabbit anti-horse IgG and their respective serum controls.

Sh Ah IgE and Rb Aeq IgG and their respective serum controls over the initial 360 minute period are shown graphically in Figure 12.2.

(a) Anti IgE: Following I/D inoculation of Sh Ah IgE a discrete wheal-like reaction was apparent within 30 minutes in all 6 horses, the diameter of which was 5 or more mm greater than that of the initial I/D bleb. These reactions continued to increase in size for up to 360 minutes, and, with the exception of horse 6, had dispersed by 24 hours. In horse 6 a diffuse raised reaction was evident at both the challenge and control sites at 24 hours (Fig. 12.1). Comparable responses did not occur after sheep serum challenge of horses 1 to 5 although the initial injection bleb remained apparent for up to 2 hours. In horse 6 a reaction was apparent at the control site at 360 minutes (Fig. 12.1), although this response was less marked than at the Sh Ah IgE site.

(b) Anti-horse Immunoglobulins: Following I/D inoculation of Rb Aeq IgG no increase in diameter of the injection bleb was apparent for 1-2 hours (Fig. 12.2), although a marked response was apparent by 360 minutes. The Sh Ah IgE and Rb Aeq IgG induced reactions were identical at 360 minutes (Fig. 12.1), each showing heat, sensitivity and, in some cases, dependant oedema. In 2 of the 3 Rb Aeq IgG recipients the reactions were still apparent at 24 hours as

diffuse plaque-like dermal swellings. Normal rabbit serum failed to elicit a comparable response in the three recipient horses, although a diffuse swelling was observed 24 hours after rabbit serum challenge of horse 6 (Fig. 12.1).

I/D challenge with Gt Aeq IgG(T) and control goat serum resulted in wheal-like responses which increased in diameter for up to 360 minutes in both cases.

I/D challenge with Sw Aeq Lc and Rb Aeq WS resulted in a wheal-like response at 120 minutes, which in the latter case increased markedly up to 360 minutes, and was still present in horse 1 as a discrete wheal-like lesion 48 hours after challenge. In these recipients no reactions resulted from challenge with pig or rabbit serum controls.

(ii) Dermal Biopsies

The histopathological findings of the dermal biopsies taken after challenge with Sh Ah IgE and Rb Aeq IgG and their respective controls are summarized in Table 12.2.

(a) Anti IgE: In comparison with normal horse dermis (Fig. 12.3,a) biopsies taken 30 minutes after I/D inoculation of Sh Ah IgE showed a vigorous vascular reaction involving the venules and precapillaries of the middle and deep dermis (Figs. 12.3, b and c). Accompanying the vasodilation and oedema the endothelial cellsin some of the vessel walls

TABLE 12.2.

SUMMARY OF HISTOPATHOLOGICAL FINDINGS OF SKIN BIOPSIES TAKEN 30 MINUTES, 4 HOURS AND 24 HOURS AFTER I/D CHALLENGE WITH Sh AhIgE AND Rb Aeq IgG AND THEIR RESPECTIVE SERUM CONTROLS.

Horse	Challenge	Biopsy Time	Dermal [a] Oedema	Vasculitis [b]	CELLULAR INFILTRATION				CELL TYPE		
					Intra [c] -vascular	Trans-[d] mural	Peri- [e] vascular	Collagen-[f] ous	Neutro-phils	Eosino -phils	Mono-nuclear
4	ShAhIgE	30 min.	2+	1+	3+	3+	1+	-	1+	-	-
		4 hours	2+	1+	1+	2+	3+	3+	3+	-	2+ *
		24 hours	-	-	-	-	1+	-	1+	-	1+ **
	Sheep Serum	30 min.	1+	-	-	-	-	-	-	-	-
		4 hours	-	-	-	-	-	-	-	-	-
		24 hours	1+	-	-	-	1+	1+	1+	-	2+ **
5	ShAhIgE	30 min.	2+	1+	2+	3+	-	-	1+	-	-
		4 hours	4+	1+	1+	2+	4+	3+	3+	-	1+ *
		24 hours	2+	1+	-	-	3+	2+	1+	1+	3+ **
	Sheep Serum	30 min.	1+	-	-	-	-	-	-	-	-
		4 hours	1+	-	-	-	1+	1+	2+	-	1+ *
		24 hours	1+	1+	-	-	1+	1+	1+	1+	3+ ***
7	RbAeqIgG	30 min.	-	-	-	-	-	-	-	-	-
		4 hours	3+	1+	2+	2+	3+	-	5+	-	1+ **
		24 hours	3+	(2-3+)	-	2+	5+	5+	3+	-	1+ ***
	Rabbit Serum	30 min.	1+	-	-	-	-	-	-	-	-
		4 hours	-	-	-	-	-	-	-	-	-
		24 hours	1+	1+	-	-	2+	1+	6+	-	1+ **
8	RbAeqIgG	30 min.	-	-	1+	-	-	-	1+	-	-
		4 hours	3+	(2-3+)	1+	3+	4+	2+	4+	-	1+***
		24 hours	4+	(2-3+)	-	3+	5+	5+	3+	-	1+***
	Rabbit Serum	30 min.	1+	-	-	-	-	-	-	-	-
		4 hours	1+	-	-	-	-	-	-	-	-
		24 hours	1+	1+	-	-	2+	3+	5+	-	1+ **

KEY (i) a. Disruption of collagen bundles in dermis with accumulation of proteinaceous fluid in the intestitial spaces. Occasional lymphatic distension.

b. 1+ - Endothelial swelling and vacuolation

2+ - Degeneration of the vascular endothelium

3+ - Frank perivascular necrosis.

c. Clumping of cells within dermal blood vessels.

d. Cells within the dermal vessel walls.

e. Cells grouped around dermal blood vessels.

f. Cellular infiltration of dermal interstitium unassociated with blood vessels:-

* Mononuclear cells predominantly lymphocytes.
** Mononuclear cells predominantly lymphocytes with occasional macrophage.
*** Mixed mononuclear cells, predominantly macrophages.

(ii) The severity of dermal oedema and cellular infiltration is graded from - (absent) to 5+ (maximum severity). The relative numbers of the individual cell types in the biopsy section are similarly graded.

appeared swollen, with pale, occasionally pyknotic nuclei (Fig. 12.3,c) and there was marked intravascular accumulation and transmural migration of neutrophil leucocytes. Eosinophil leucocytes were not observed in the 30 minute biopsies from either horse. In the control biopsies comparable vascular and cellular reactions were not apparent (Fig. 12.3,d).

Perivascular intact mast cells were apparent throughout the dermis in the normal unchallenged skin of horse 4 (Fig. 12.3,e), whereas 30 minutes after challenge with Sh Ah IgE mast cells from the same animal showed swelling, disruption and pericellular dispersal of their metachromatic granules (Fig. 12.3,f). 30 minutes after sheep serum challenge the dermal mast cells did not differ from those in normal skin (Fig. 12.3,g). Similar mast cell changes were observed 30 minutes after I/D Sh Ah IgE and sheep serum challenge of horse 5. In both test biopsies, the mast cell changes occurred exclusively in the middle and deep dermis, although the cells were more numerous in the upper sub-epidermal region.

Four hours after challenge with Sh Ah IgE the cellular reaction was predominantly perivascular (Fig. 12.3, h). Transmural neutrophil migration was still occurring at 4 hours and there was evidence of cytoplasmic swelling, vacuolation and pyknosis of the vascular endothelial cells (Fig. 12.3,i). Oedema

was still apparent and in horse 5 there was perivascular lymphatic distension. The control biopsies at this time showed essentially normal dermis, although a limited perivascular, mainly polymorphonuclear, infiltrate was observed associated with some of the blood vessels in the deep dermis of horse 5 (Fig. 12.3,j).

In both test and control biopsies 4 hours after challenge, the morphology of the mast cells did not differ from those in normal skin.

The dermal biopsies 24 hours after Sh Ah IgE and normal sheep serum challenge in both horses showed a predominantly mononuclear perivascular and collagenous cellular reaction which was more marked in horse 5 (Fig. 12,3,k). The dominant cell types were lymphocytes, with the occasional macrophage and eosinophil. Both control biopsies showed a limited perivascular inflammatory response, possibly the result of foreign protein challenge (Fig. 12.3, l).

(b) Anti-horse IgG: Thirty minutes after I/D challenge with Rb Aeq IgG the biopsy from horse 7 showed normal dermis while that from horse 8 showed limited intravascular accumulation of neutrophil leucocytes affecting only a few vessels (Fig. 12.3,m). No oedema was present in either biopsy. Biopsies from the serum challenge control sites from both horses showed no changes.

Four hours after Rb Aeq IgG challenge, biopsies from both horses showed a vigorous cellular reaction in and around dilated and occasionally thrombosed precapillaries and venules in the middle and deep dermis. There was transmural migration and perivascular accumulation of predominantly neutrophil leucocytes, many of which appeared pyknotic and dying (Fig. 12.3, n and o). The majority of the infiltrating mononuclear cells were macrophages. Dermal oedema, haemorrhage and accumulation of homogeneous eosinophilic material was present in both biopsies, and in the biopsy taken from horse 8 focal areas of necrosis around obliterated blood vessels were apparent in the deep dermis (Fig. 12.3, n and p). In the control biopsies the appearance of the dermal vasculature and interstitium 4 hours after challenge was essentially normal (Fig. 12.3q), with the exception of some interstitial oedema apparent in horse 8.

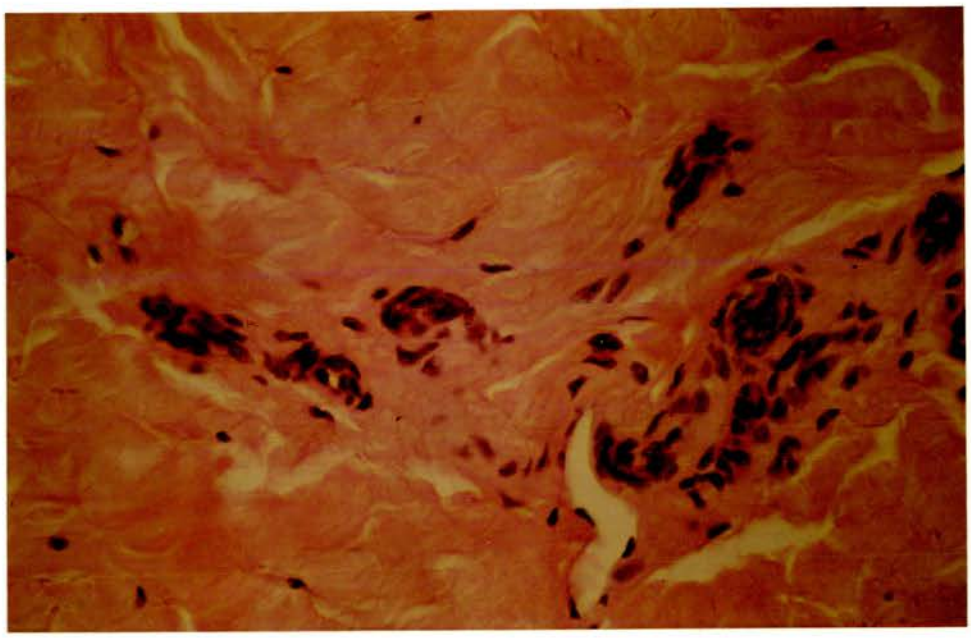
24 hours after I/D Rb Aeq IgG challenge of both horses there was marked cellular infiltration of the dermis accompanied by extensive interstitial oedema, haemorrhage and accumulation of homogenous eosinophilic material (Fig. 12.3,r). As in the 4 hour biopsy, many of the blood vessels appeared obliterated by overt necrosis. A mixed population of cells was present in the biopsies from both horses, and although the relative numbers of mononuclear cells had increased in comparison to the 4 hour biopsy, the

FIG. 12.3.

Histology of sheep anti-human IgE serum and rabbit anti-horse IgG serum induced reactions in horse skin.

(a) Normal horse dermis. H & E, x 440.

(b) Sheep anti-human IgE, 30min. H & E, x 440.



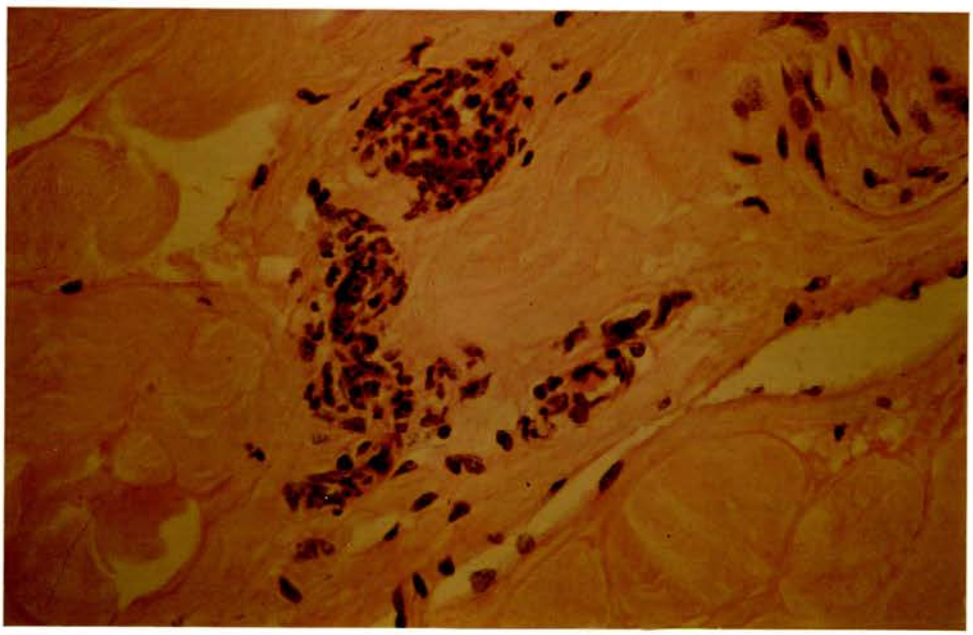
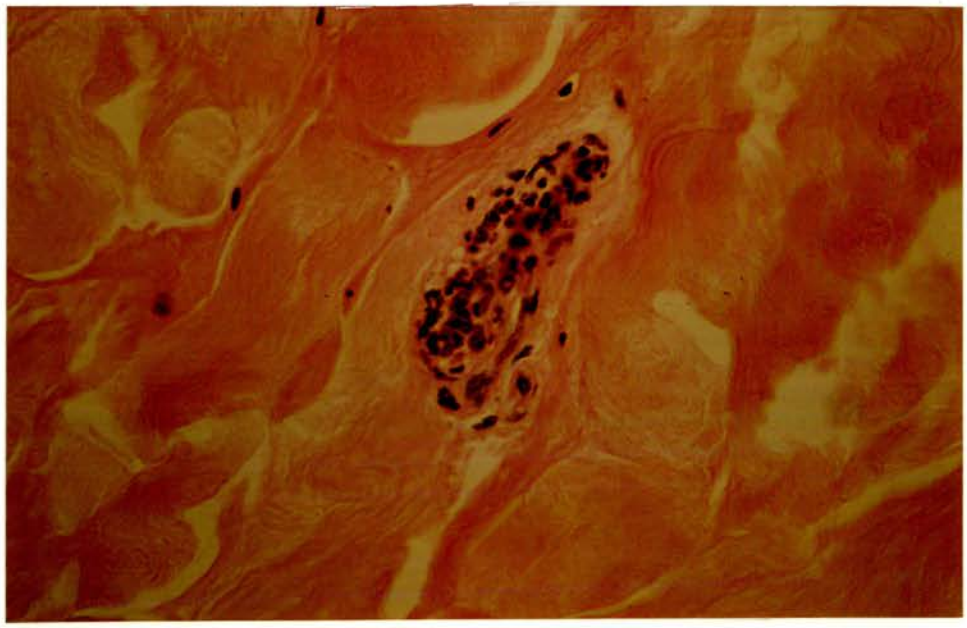


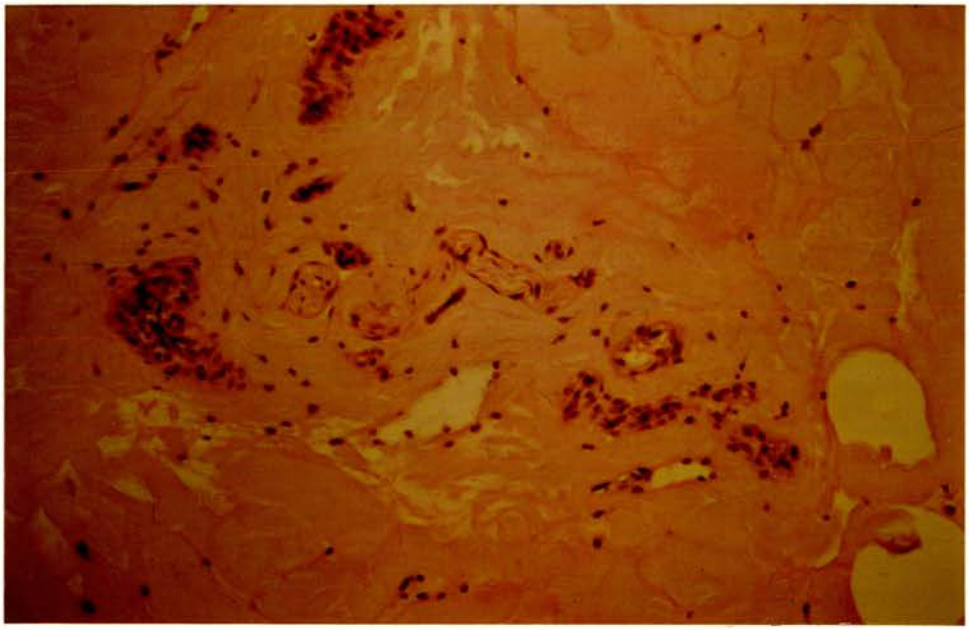
FIG.12.3.(cont'd.)

(c) Sheep anti-human IgE, 30min. H & E, x 440.

(d) Sheep serum, 30min. H & E, x 180.

(e) Normal horse dermis. Tol. Blue (pH 4.2), x 440. (Mast cells arrowed).





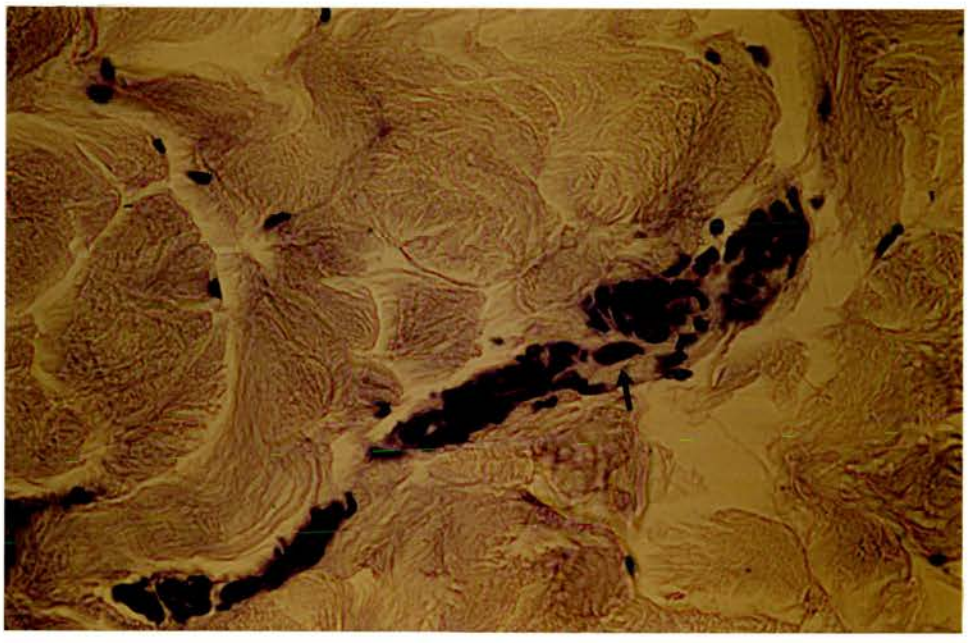
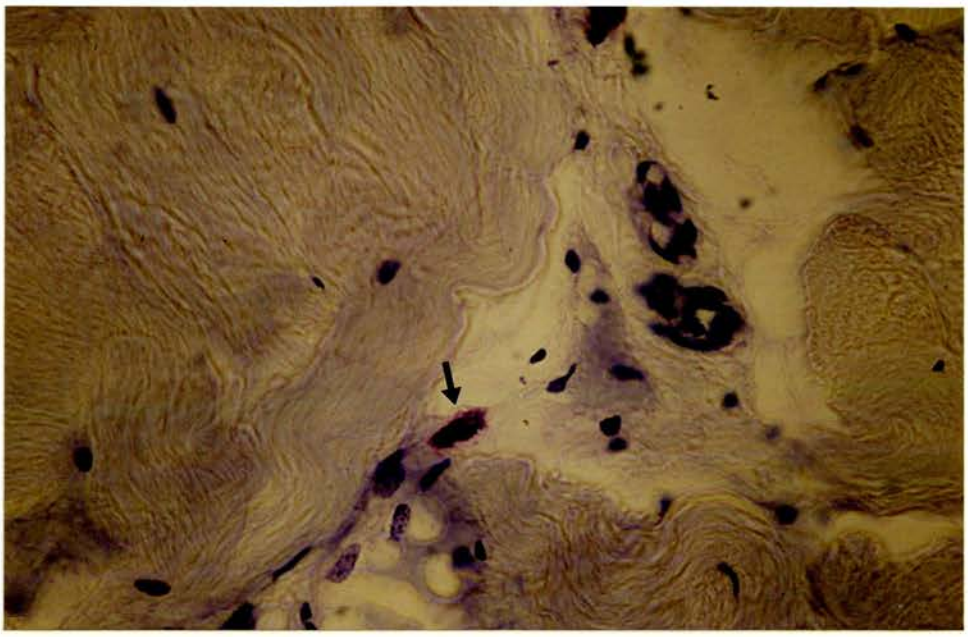


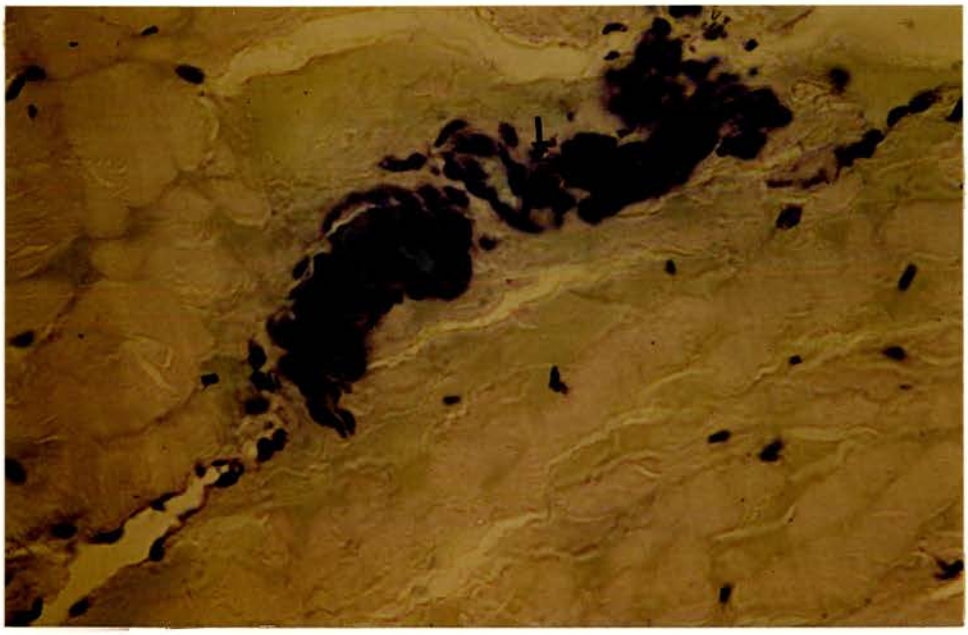
FIG.12.3(cont'd.)

(f) Sheep anti-human IgE, 30 min. Tol. Blue (pH 4.2), x 440. (Mast cells arrowed)

(g) Sheep serum, 30 min. Tol. Blue (pH 4.2), x 440. (Mast cells arrowed)

(h) Sheep anti-human IgE, 4 hours. H & E, x 180.





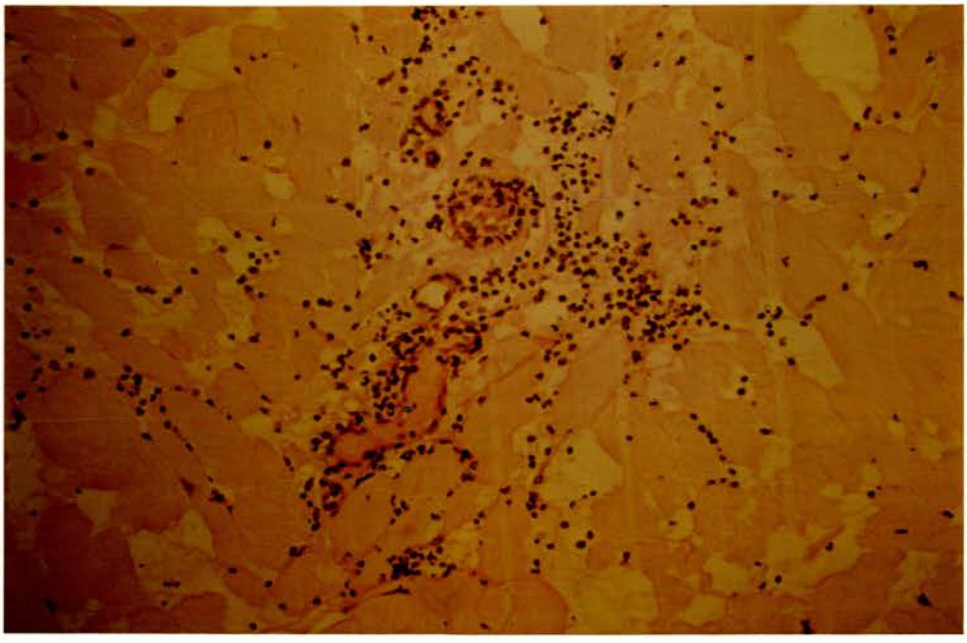
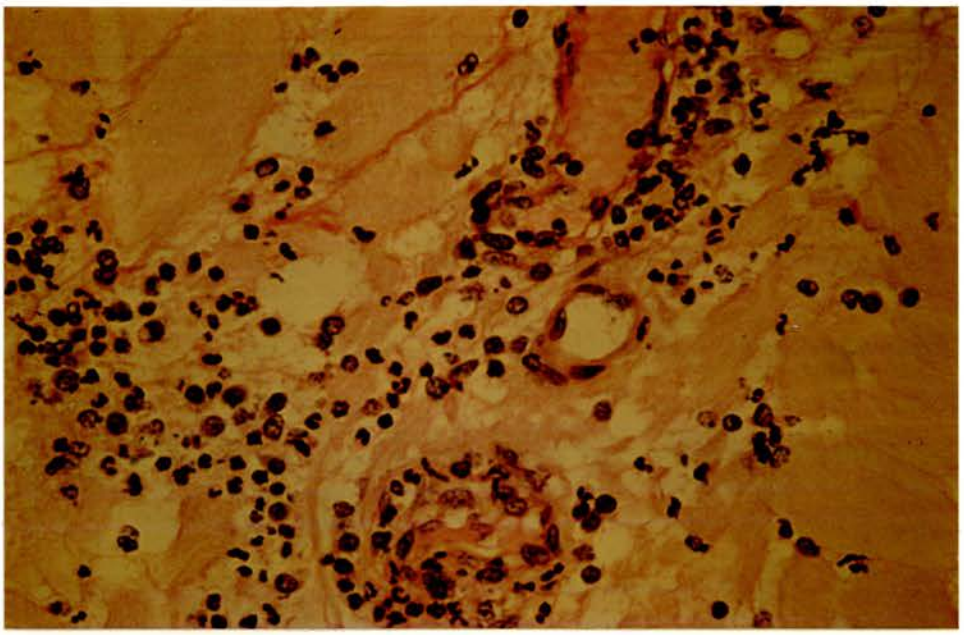


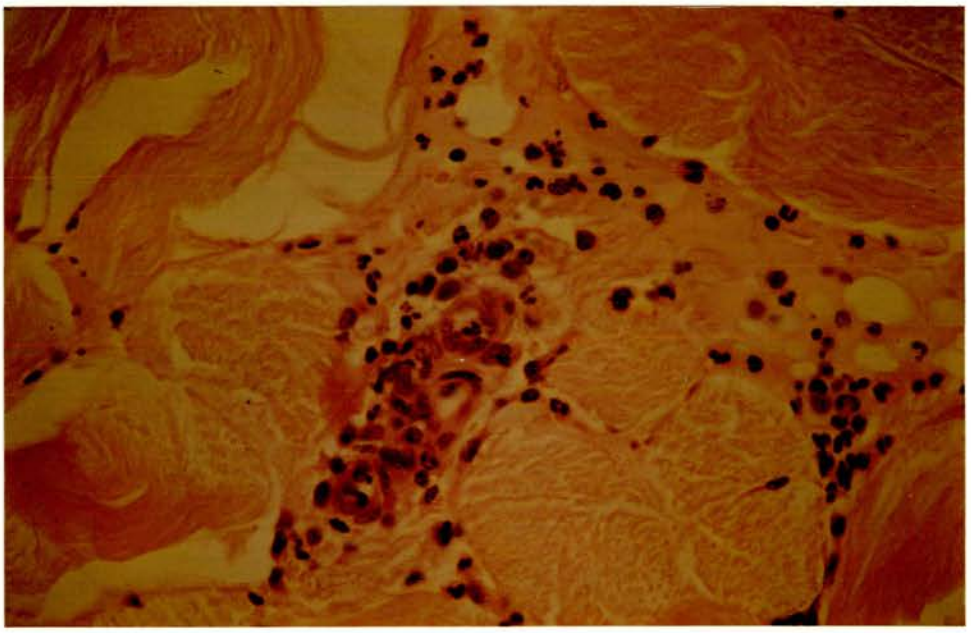
FIG.12.3 (cont'd.)

(i) Sheep anti-human IgE, 4 hours. H & E, x 440.

(j) Sheep serum, 4 hours. H & E, x 440.

(k) Sheep anti-human IgE, 24 hours. H & E, x 180.





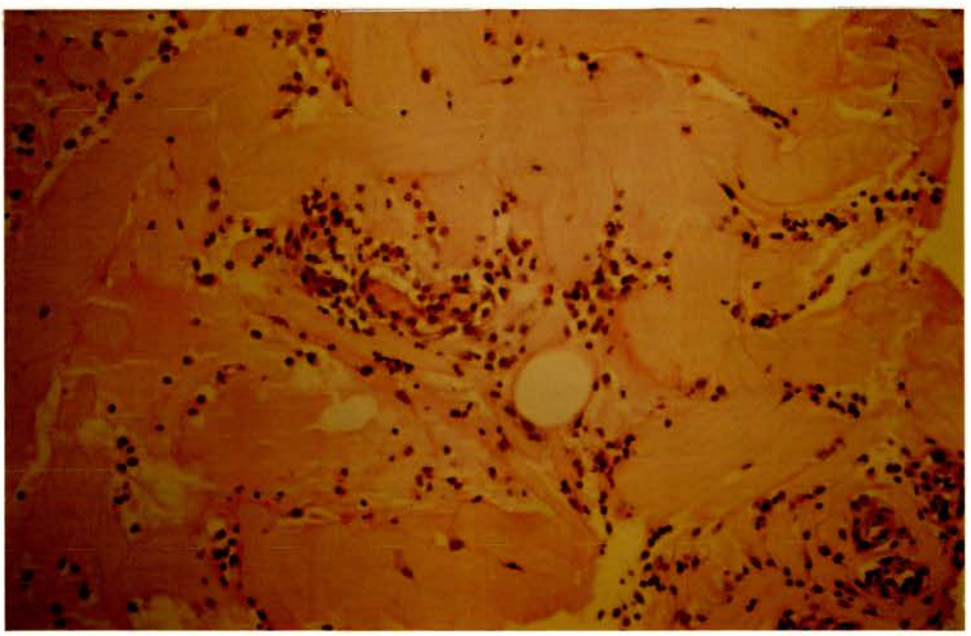
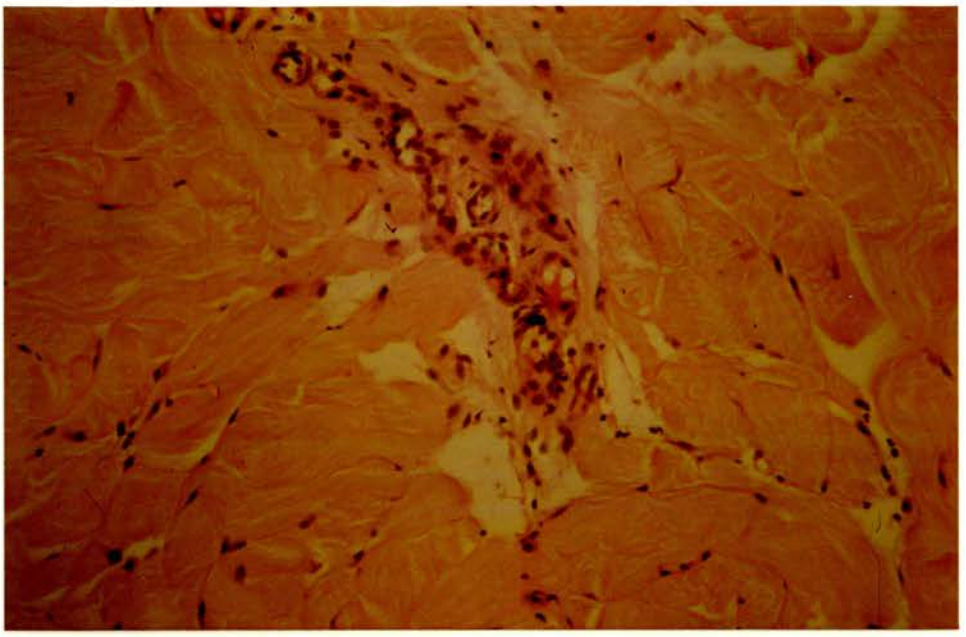


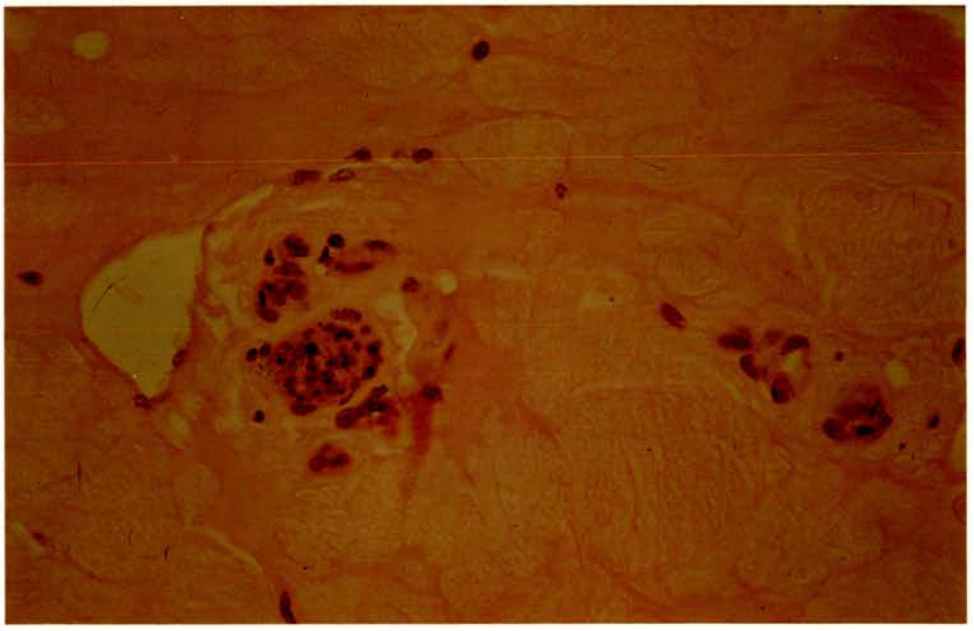
FIG. 12.3 (cont'd)

(l) Sheep serum, 24 hours. H & E, x 180.

(m) Rabbit anti-horse IgG, 30 min. H & E, x 440.

(n) Rabbit anti-horse IgG, 4 hours. H & E, x 180.





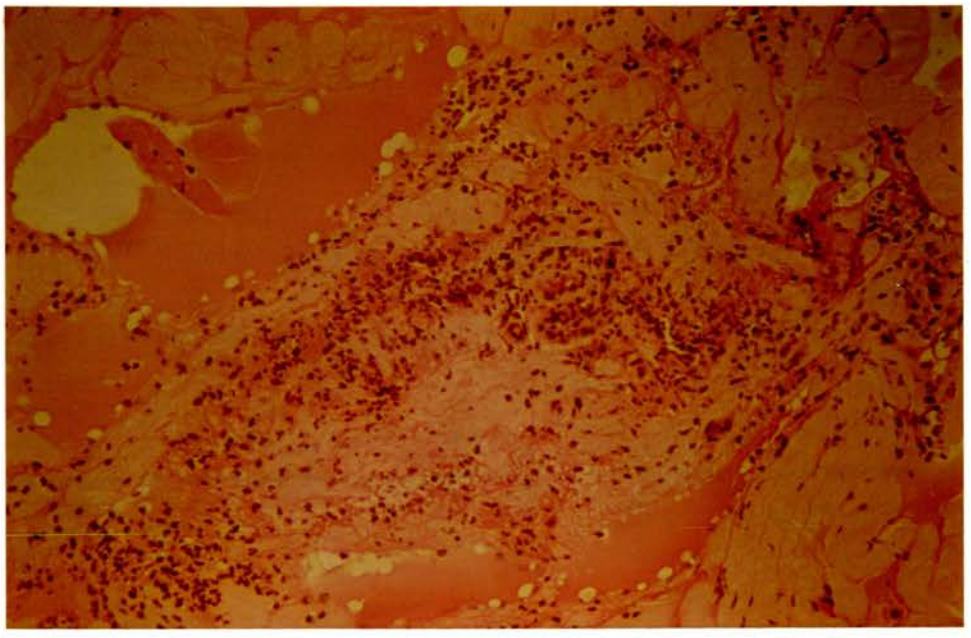
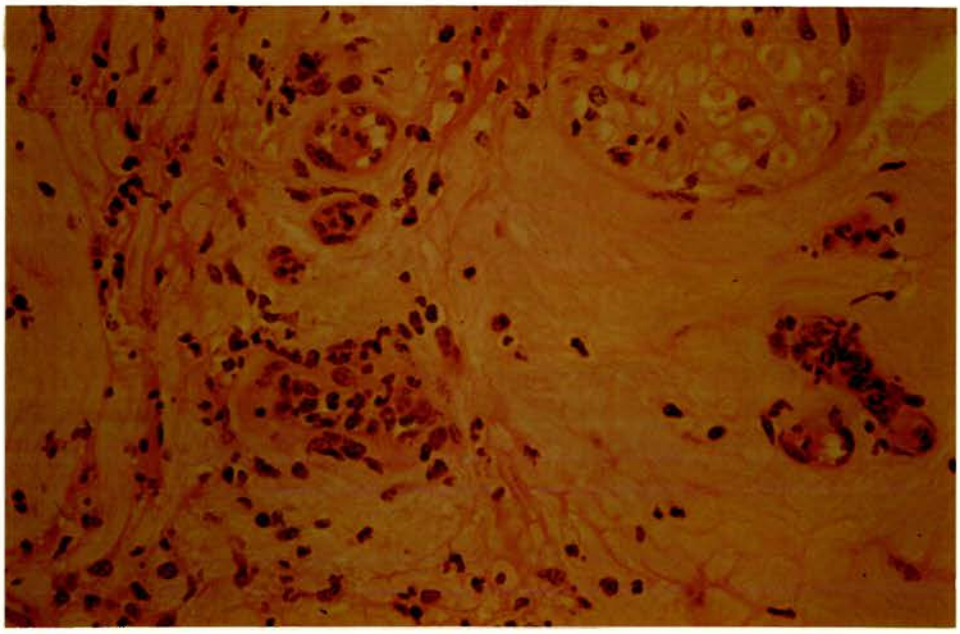


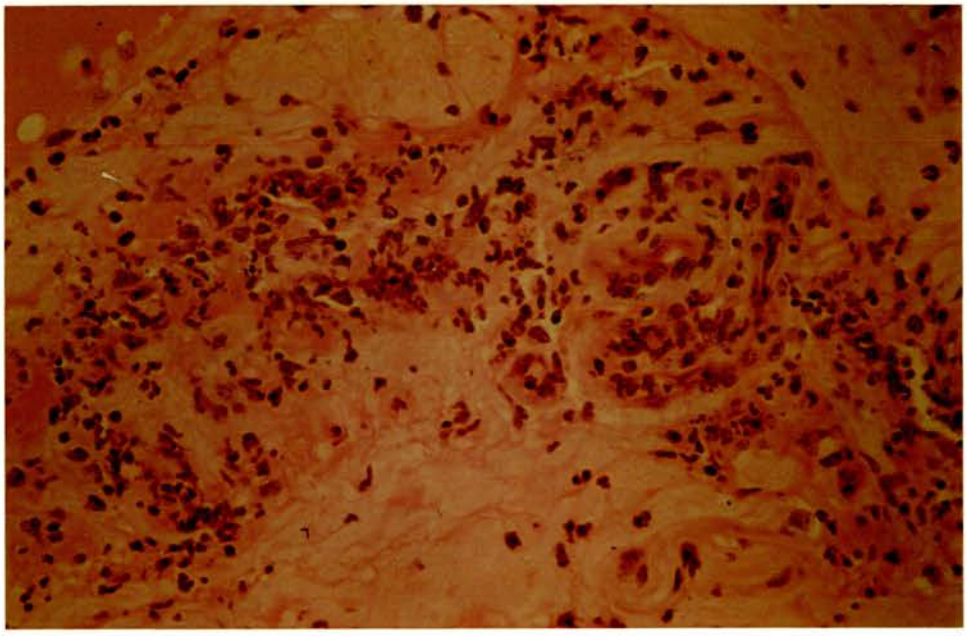
FIG. 12.3 (cont'd).

(o) Rabbit anti-horse IgG, 4 hours. H & E, x 440.

(p) Rabbit anti-horse IgG, 4 hours. H & E, x 440.

(q) Rabbit serum, 4 hours. H & E, x 180.





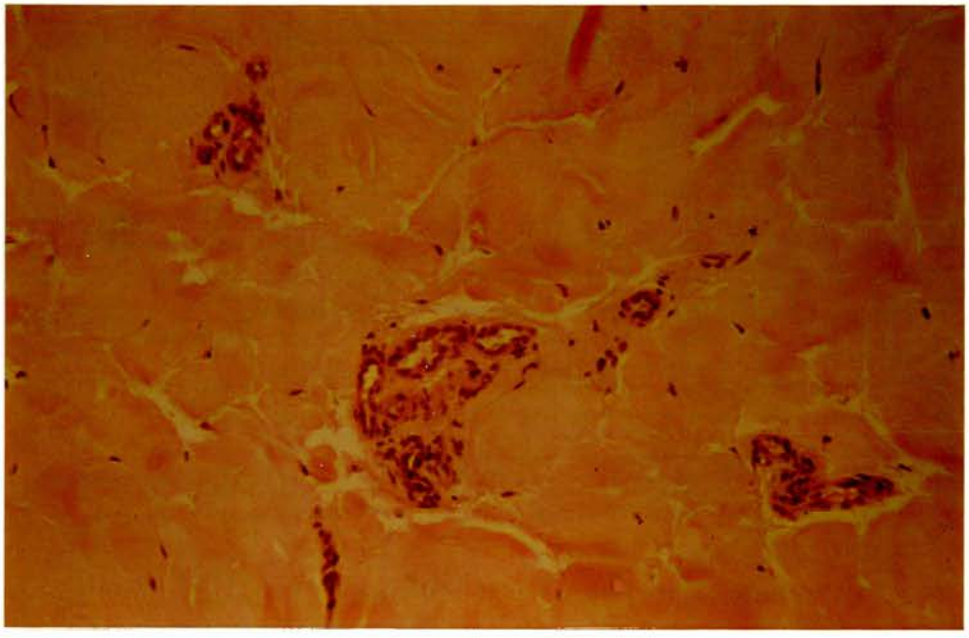
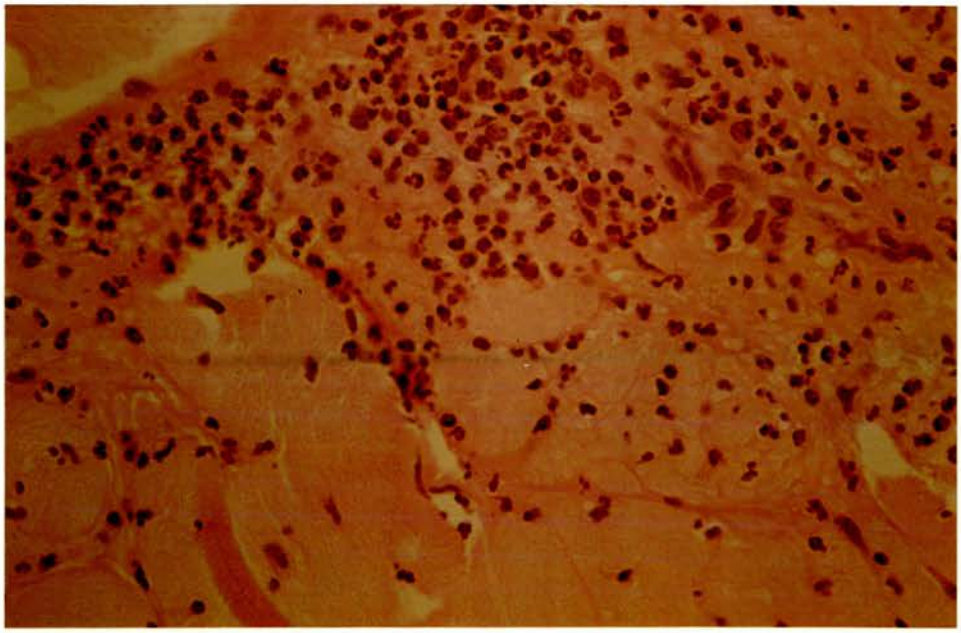
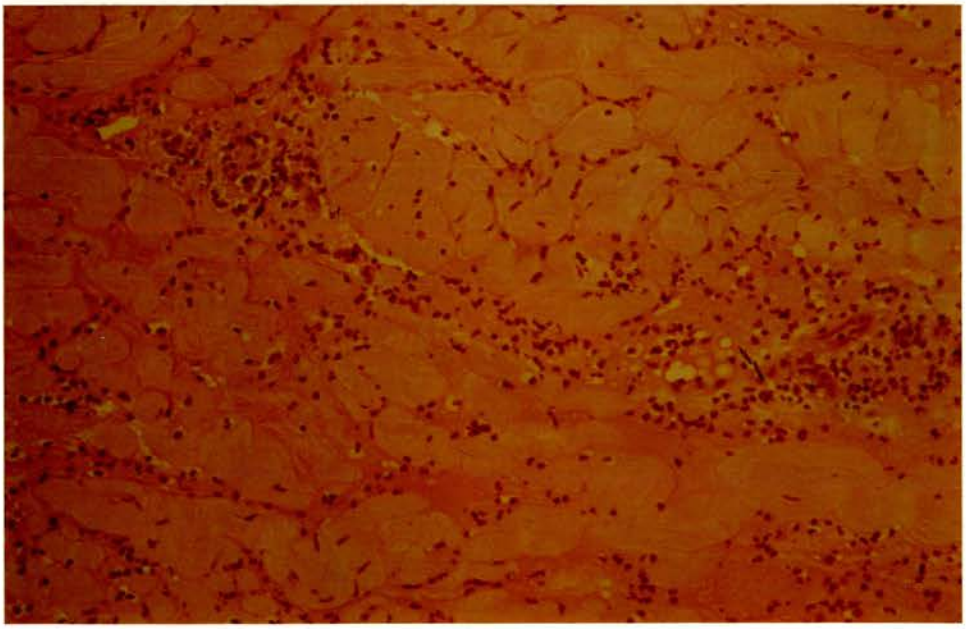


FIG. 12.3.(cont'd.)

(r) Rabbit anti-horse IgG, 24 hours. H & E, x 440.

(s) Rabbit serum, 24 hours. H & E, x 180.





cells remained predominantly neutrophils. Examination of both 24 hour control biopsies showed a diffuse, predominantly neutrophil response with evidence of limited vascular degeneration (Fig. 12.3s). Relatively fewer macrophages were present in the controls in comparison to the test reactions. The histopathological features of the biopsy at this stage were those of acute inflammatory response, resulting possibly from rabbit serum challenge.

No significant changes in the dermal mast cell morphology was observed 30 minutes after anti-IgG challenge, although by 4 hours mast cells were inapparent in the disrupted dermis.

12.2.4. DISCUSSION

These observations on I/D Sh Ah IgE inoculation in the horse indicate that the antiglobulin is capable of inducing an immediate and prolonged response similar to the response elicited using the R.S.D. sera - C. pulicaris antigen P-K model (10.3). Furthermore, the anti-IgE induced response appears to be morphologically similar to that reported following I/D inoculation of histamine and a number of artificial mast cell degranulating compounds, including compound 48/80 and Concanavalin A (Mansman and Mansman, 1975; Hodgin et al., 1978), suggesting that the response is a true reversed anaphylaxis associated with antiglobulin induced basophil degranulation. By analogy with RCA responses in primate skin (Ishizaka and Ishizaka, 1969) the anti-human IgE induced response in

horse skin will be dependant upon interaction of the $F(ab^1)_2$ fragment of the antiglobulin with the antigenic determinants on the Fc fragment of the horse homocytotropic antibody.

Although the RCA response induced in primates (Ishizaka and Ishizaka, 1969) and dogs (Halliwell et al., 1972) by I/D inoculation of anti-human IgE are typically transient wheal and flare responses, both Dolovich et al., (1973) and Umemoto et al., (1976) reported prolonged LCAR-like responses after I/D inoculation of anti-human IgE in atopic and non-atopic human subjects. These reversed LCAR-like responses were morphologically similar to the anti-human IgE responses observed in the present study.

Both the 30 minute and 4 hour biopsies of sites challenged with Sh Ah IgE show many of the histological features of the PCA response in guinea pigs and primates (Fisher and Cooke, 1957; Parish, 1965). These are an initial vasodilation of the dermal venules with concomitant oedema, rapidly followed by swelling of the vascular endothelium and intravascular aggregation and transmural migration of predominantly polymorphonuclear leucocytes. Four hours after this initial reaction there is a predominantly perivascular polymorphonuclear leucocyte infiltration of the oedematous dermis with scattered traces of endothelial degeneration. However the RCA-like reactions in the present study appear to develop more slowly than the

PCA responses in laboratory animals. This is shown by the presence of transmural leucocyte migration in blood vessels in the 4 hour anti-IgE biopsies, a feature not observed in 4 hour PCA biopsies.

The extent of the oedema, cellular infiltration and vascular damage observed in the 4 hour anti-IgE biopsies in the present series is similar to that reported by Solley et al., (1976) in 4 hour biopsies of LCARs elicited in human skin during P-K testing of ragweed sensitive human serum. These authors reported approximately 1:1 ratios of polymorphonuclear and mononuclear cells in the infiltrate, in contrast to the predominantly polymorphonuclear cell infiltrate in the present series. However, Dolovich et al., (1973) earlier reported a predominantly polymorphonuclear leucocyte infiltration of LCAR biopsies in non-atopic subjects after challenge with anti-human IgE or anti-$F(ab^1)_2$ of human IgE.

The crucial role of circulating basophils and tissue mast cells in the anaphylactic response is well established (Keller, 1966), and Bloom and Chakravarty (1970) described a series of morphological changes occurring in sensitized tissue mast cells following incubation with antigen. These are primarily vacuolation and extrusion of the cytoplasmic granules. Although Kruger (1976) has shown that these changes are not necessary for histamine release, mast cell degranulation was observed in the 30 minute anti-IgE

biopsy sections in the present series. However the difficulties in interpretation of changes in mast cell staining affinity, morphology and numbers have been discussed by Head (1965) and are increased by the apparent susceptibility of horse mast cells to disruption during fixation and sectioning (Hodgin et al., 1978; Baker and Quinn, 1978). Nevertheless, the mast cell changes apparent in the 30 minute anti-IgE biopsies in the present study were absent from the control and normal skin biopsies.

Although the histopathological features of RCA-like responses in the present study indicate the participation of both leucotactic and vasoactive substances in the genesis of the response, a major difference between these responses and previous reports of dermal anaphylactic responses in the horse is the absence of eosinophil leucocytes (Riek, 1954; Baker and Quinn, 1978). Since eosinophil infiltration is considered pathognomonic of local anaphylaxis (Broder, 1979), their absence from the heterologous RCA-like responses in the horse is puzzling. However, although eosinophil infiltration is a feature of anti-IgE induced LCARs in non-atopic humans (Dolovich et al., 1973), eosinophilia of the middle and deep dermis was not observed 2 hours after challenge of non-atopic humans with compound 48/80 at a dosage shown by Solley et al., (1976) to result

in an LCAR-like response (Atkins, Green and Burton, 1973). In the horse, Mansman and Mansman (1975) observed LCAR-like responses following I/D injection of compound 48/80 although, histologically, eosinophilia of the dermis was not apparent. Yet Hodgin, et al., (1978) reported eosinophil infiltration in biopsies of the wheal-like responses apparent 4 hours after I/D inoculation of Concanavalin A in adult horses. Concanalvalin A and compound 48/80 are non-cytolytic releasers of vasoactive substances from mast cells (Siraganian and Siraganian, 1975; Kasimierczak and Diamant, 1978), although in contrast to Concanavalin A, compound 48/80 is a poor releaser of histamine from human and horse blood basophils (Siraganian and Siraganian 1975; Diamant, 1978; Kings and de Weck, 1980).

Histamine is potently eosinophilotactic in horse skin (Archer, 1960), but it is only one of a number of vasoactive and leucotactic compounds released during systemic and local anaphylaxis in the horse, which include 5-HT, kinins and prostaglandins (Eyre and Lewis 1973, Eyre 1976). These latter compounds have not been shown to be eosinophilotactic (Broder, 1979). Although the relative individual importance of these vasoactive mediators in the genesis of dermal anaphylaxis in the horse is not known, Burka et al., (1976) have published preliminary data which shows that in the horse the relative percentages of the

available mediators in a tissue preparation which are released after immunological or non-immunological basophil disruption varies with the nature of the disruptive challenge. Furthermore, there is evidence to indicate that the histamine releasing activity of chemical mast cell 'degranulating' agents vary between tissues and species (Kazimierczak and Diamant, 1978). Hypothetically therefore, compound 48/80 and antihuman IgE inoculated into horse skin may result in the release of a spectrum of vasoactive and cytotactic agents different from that released by direct antigen challenge of sensitized skin and may provoke a typical anaphylactic response without histamine dependent eosinophilia.

The reaction elicited by I/D inoculation of Rb Aeq IgG in the horse differs from the Sh Ah IgE induced RCA responses in a number of ways. Most significantly the former response was virtually inapparent at 30 minutes although by 4 hours an oedematous dermal wheal was apparent which histologically showed a severe necrotizing vasculitis. This contrasts with the infiltrative perivascular cellular response observed in the 4 hour anti-IgE biopsy. By 24 hours, the anti-IgG response in 2 of the 3 recipient horses was still apparent as a diffuse plaque-like lesion, which histologically showed evidence of widespread peri-vascular necrosis and interstitial cellular infiltration in contrast to the receding anti-IgE reactions at 24 hours.

The response to I/D Rb Aeq IgG challenge is a model of the reversed passive Arthus response (RPAR) (Anderson, 1976) classically elicited by I/D antibody inoculation followed immediately by intravenous antigen challenge, the antigen in this case being endogenous circulating IgG. Morphologically and histologically the RPARs in this series show the characteristic features of a Type III hypersensitivity reaction as originally described by Arthus and Breton (1903) and recently reviewed by Ranadive and Movat (1979). These are; vasodilatation and intravascular accumulation of platelets and leukocytes along with thrombosis, oedema and accumulation of eosinophilic proteinaceous fluid. Initially the cell population is primarily polymorphonuclear leucocytes although later mononuclear cells become more prominant. Focal perivascular necrosis is a feature of the later stages of the response.

Schatzman, et al., (1973) recorded typical Arthus-type histopathological changes following I/D antigen challenge of a horse experimentally sensitized to bovine gamma globulin. Although the dermal RPAPs in the present series were slower to develop and were histologically less severe than the direct Arthus model described by Schatzman et al., differences in rate of onset and intensity of RPARs and direct Arthus responses in guinea pigs have been described (Uriuhara and Movat, 1964).

RPAR-like responses were also observed after I/D inoculation of anti-whole horse serum and anti-horse light chain serum. The 48 hour reaction observed in horse 1 following anti-whole horse serum inoculation probably reflects the severity of the necrotizing response in this individual animal since frank morphological responses 48 hours after challenge in experimental active Arthus reactions are commonly observed (Ranadive and Movat, 1979). However, the failure of anti-IgG(T) to induce a response is unexpected, but may be the result of low circulating IgG(T) 'antigen' levels in this horse, the levels of circulating IgG(T) in adult horses being approximately 13% those of IgG (Rouse, 1971).

Since the Sh Ah IgE induced RCA-like responses in horse skin described in the present study appear to result from antigenic cross reactivity of the anti-globulin with basophil bound homocytotropic antibody, the morphological similarity of the RCA and RPAR-like responses at 4 hours might suggest that the later stages of the former response may be an Arthus reaction mediated by anti-IgE-homocytotropic antibody complexes. However, the histopathological differences between the two responses indicate that the reactions occur independently of one another and that the prolonged RCA-like responses is primarily dependant upon common Fc antigenic determinants on the horse homocytotropic antibody and human IgE.

12.3. ABSORPTION OF THE RCA-LIKE ACTIVITY OF ANTI-HUMAN IgE IN HORSE SKIN WITH P-K POSITIVE R.S.D. SERUM.

12.3.1. INTRODUCTION

A number of authors have examined the ability of anti-human IgE to neutralize the P-K or PCA activity of the reaginic serum of other species (Halliwell et al., 1972; Doyle, 1973; Barratt, 1972), and Ishizaka and Ishizaka (1968) have also demonstrated the neutralization of the RCA activity of anti-IgE in monkey skin by human serum.

Since anti-human IgE is able to elicit RCA-like responses in horse skin, then the presence of excess anti-IgE in an antiglobulin-reaginic serum incubate may influence the results of P-K neutralisation experiments. Thus the simpler alternative of RCA neutralisation by P-K positive R.S.D. serum was examined as a means of further identifying antigenic cross reactivity of human IgE and horse homocytotropic antibody.

12.3.2. MATERIALS AND METHODS

The neutralization procedure was based upon the methods of Ishizaka and Ishizaka (1968) and Halliwell, et al., (1972).

0.3ml pooled P-K positive R.S.D. serum was added to an equal volume of Fc specific sheep anti-human IgE[1] and incubated for 4 hours at 37°C. The mixture was centrifuged at 3500g for 20 minutes and the top 0.4ml

1. Nordic Immunological Laboratories, Maidenhead, England.

of supernatant was removed. Doubling dilutions were prepared in sterile 0.01M PBS (pH 7.4) up to $\frac{1}{256}$, corresponding to an anti-IgE titre of $\frac{1}{512}$. As controls, anti-IgE serum was incubated with both PBS and normal horse serum, and after centrifugation doubling dilutions were prepared up to $\frac{1}{256}$.

0.1ml of each dilution was injected I/D into the lateral cervical region of an experimental horse. $^{1}/_{4}$ dilutions of R.S.D. serum, normal horse serum and normal sheep serum in PBS were also injected as negative controls. Positive RCA-like responses were indicated by an increase in horizontal wheal diameter of $\geq$5mm over the initial 30 minutes.

12.3.3. RESULTS

The RCA activity of the test and control dilutions are shown in Table 12.3. A positive response was elicited up to a dilution of $\frac{1}{128}$ of the Sh Ah IgE-PBS incubate, indicating an RCA titre of $\frac{1}{256}$ for whole anti-IgE serum. Following incubation with R.S.D. sera the RCA titre of whole anti-IgE serum is reduced to $\frac{1}{32}$, indicated by detectable RCA activity of the antiglobulin - R.S.D. serum incubate up to a dilution if $^{1}/16$. Incubation with normal serum reduced the RCA titre of whole anti-IgE serum to $^{1}/128$.

At all three negative control sites increases in horizontal diameter of the injection bleb of between 1-2mm occurred over the initial 30 minute period.

TABLE 12.3.

THE EFFECT OF INCUBATION WITH POOLED P-K+ve R.S.D. SERUM ON THE RCA ACTIVITY OF SHEEP ANTI-HUMAN IgE IN HORSE SKIN.

INCUBATE \ DILUTION	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Sh.AhIgE/PBS	+	+	+	+	+	+	+	-
Sh.AhIgE/R.S.D. serum	+	+	+	+	-	-	-	-
Sh.AhIgE/normal serum	+	+	+	+	+	+	-	-

Positive RCA responses indicated by an increase in wheal diameter of ≥ 5mm over initial 30 mins.

12.3.4. DISCUSSION

The eight fold reduction in RCA titre of whole anti-human IgE serum after incubation with the R.S.D. serum is strongly suggestive of the specific absorption of the antiglobulin by horse homocytotropic antibodies. The reduction in RCA titre from $^{1}/_{256}$ - $^{1}/_{128}$ following incubation with normal horse serum may indicate the presence of homocytotropic antibodies in normal horse serum, although a two fold reduction in titre in 'in vivo' neutralisation experiments lies within the limits of experimental variation (Halliwell et al., 1972).

12.4. IMMUNOFLUORESCENT LABELLING OF CELL BOUND ANTI-HUMAN IgE IN HORSE SKIN AND DUODENUM.

12.4.1. INTRODUCTION

Fluorochrome conjugated antisera against homologous reagin has been used to demonstrate the association of the reagin with tissue mast cells and plasma cells in man (Hubscher, Watson and Goodfriend, 1970; Tada and Ishizaka, 1970) and the rat (Mayrhofer, Bazin and Gowans, 1976), and with dermal mast cells in the dog (Halliwell, 1973). Recently Healey and Gaafar (1977) demonstrated intracellular IgE in canine dermal mast

cells using fluorescein isothiocyanate (FITC) conjugated anti-human IgE.

Following the 'in vivo' demonstration of antigenic cross reactivity of human IgE and horse homocytotropic antibody, 'in vitro' confirmation of this relationship was sought by indirect immunofluorescence of horse dermis and duodenum.

12.4.2. MATERIALS AND METHODS

(i) Dermis

(a) Preparation: 8mm punch biopsies were removed under local anaesthesia from the lateral cervical region of 7 horses at sites sensitized 24 hours previously by I/D injection of 0.1ml pooled P-K positive R.S.D. serum. The biopsies were immediately frozen in liquid nitrogen cooled isopentane. 6μ sections were cut on a cryostat at -25°C, dried and fixed in 2% glutaraldehyde in 0.01M PBS (pH 7.4).

Initial experiments using 95% ethanol fixation as described by Halliwell (1973) for the preparation of cryostat sections of canine dermis of anti-IgE fluorescence resulted in loss of adhesion of the sections to the slide. This is a recognised hazard of ethanol fixation (Nairn, 1969). Glutaraldehyde has been used by Perelmutter, et al., (1972) and Simson, et al., (1977) for the preparation of rat mast cells prior to fluorochrome and peroxidase labelling. However, thorough washing of glutaraldehyde fixed sections

is necessary to eliminate the effects of auto fluoroescence (Nairn, 1969).

(b) Indirect Immunofluorescence: The fixed sections were washed in two changes of cooled PBS over a period of 30 minutes. After encircling with nail varnish the sections were overlaid with a 1/5 dilution of sheep anti-human IgE[1] in PBS and incubated at 37°C for 30 minutes in a moist chamber. The sections were washed in 3 changes of PBS over a 45 minute period and overlaid with a 1/20 dilution of FITC conjugated rabbit anti-sheep globulin serum[1] in PBS. Following incubation at 37°C for 30 minutes the sections were washed in 3 changes of PBS and the wet preparations were examined using transmitted blue light from a mercury vapour lamp with a 2mm BG 38 and a 2mm BG 12 filter.[2]

After examination, the fluorescent sections were overstained with toluidine blue (pH 4.2) using a standard technique.

Control sections included for each biopsy were treated as follows; overlaid with normal sheep serum followed by FITC conjugated anti-sheep globulin serum, overlaid with PBS and FITC conjugated anti-sheep globulin serum, overlaid with PBS alone. Control sections were washed as for the test sections.

Adjacent glutaraldehyde fixed cryostat sections

1 Nordic Immunological Laboratories, Maidenhead, England.

2 M20 microscope; Wild Instruments, Heerburg, Switzerland.

from each biopsy were stained with Haematoxylin and Eosin and Toluidine Blue (pH 4.2) using standard techniques.

(ii) Duodenum

(a) Preparation: Samples of duodenum were removed from 3 horses post mortem. After fixation in Carnoy's fixative at 4°C for 24 hours the tissue was dehydrated in xylene for 12 hours and embedded in paraffin wax. The blocks were stored at 4°C and 6μ sections were cut and dewaxed immediately before each experiment.

Since non-specific eosinophil fluorescence was encountered in early experiments, the sections were pretreated with Lendrum's carbol chromotrope for 30 minutes to convert this apple green fluorescence to orange (Johnston and Bienenstock, 1974).

(b) Indirect Immunofluorescence: The duodenal sections were treated as described in (i) above.

12.4.3. RESULTS

(i) Dermal Biopsies

Specific fluorescence was not observed in any of the control preparations in this series. The results of indirect anti-human IgE immunofluorescence and toluidine blue staining of adjacent biopsy sections are presented in Table 12.4. In 4 of the 7 sections the preparation procedure resulted in mast cell disruption and

TABLE 12.4.

RESULTS OF INDIRECT ANTI-HUMAN IgE IMMUNOFLUORESCENCE AND TOLUIDINE BLUE STAINING OF ADJACENT CRYOSTAT SECTIONS OF BIOPSIES OF R.S.D. SERUM SENSITIZED HORSE DERMIS.

Biopsy	Indirect anti-human IgE Immunofluorescence	Toluide blue pH 4.2
1	Specific cytoplasmic fluorescence of 6 cells throughout section.	Numerous intact mast cells.
2	Localised granular fluorescence.	Majority of mast cells disrupted.
3	No specific fluorescence	Majority of mast cells disrupted
4	No specific fluorescence	Numerous intact mast cells.
5	No specific fluorescence	Mast cells disrupted.
6	No specific fluorescence	Few but intact mast cells.
7	No specific fluorescence	Mast cells disrupted.

dispersal of the metachromatic granules. Of the 3 preparations with intact dermal mast cells only one (biopsy 1) showed specific cellular fluorescence. This fluorescence, shown in Figure 12.4, a and b, was cytoplasmic and in all cases an unstained peripheral nucleus was apparent. In this section a total of 6 fluorescent cells were observed, representing only a fraction of the mast cells apparent after toluidine blue staining of the adjacent section.

Toluidine Blue overstaining of the fluorescent preparations resulted in both the loss of cells and weak or ineffective metachromatic staining of the mast cells in contrast to the routinely prepared cryostat sections. However, toluidine blue overstaining of the test preparation from biopsy 1 (Fig. 12.5) demonstrated weak metachromatic staining of the single fluorescent cell shown in Figure 12.4,b. Identification of the remaining fluorescent cells on the overstained preparation was not possible.

(ii) Duodenal Preparations

Specific cytoplasmic fluorescence was observed in the test section of only 1 of the 3 preparations and was associated with perivascular cells within the lamina propria (Fig. 12.6). However, due to tissue loss and ineffective metachromatic staining the identity of these cells was not established after toluidine blue overstaining (Fig. 12.7). Cellular fluorescence was not observed in the control sections

FIG. 12.4,a.

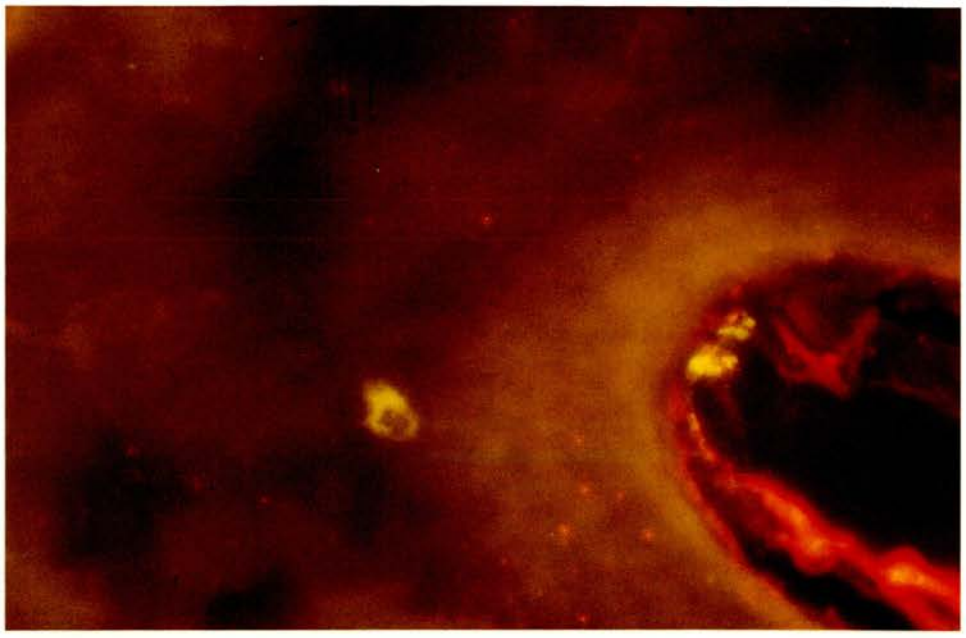
Indirect immunofluorescence of horse dermis; anti-human IgE preparation of section from biopsy 1 (Table 12.4). x440

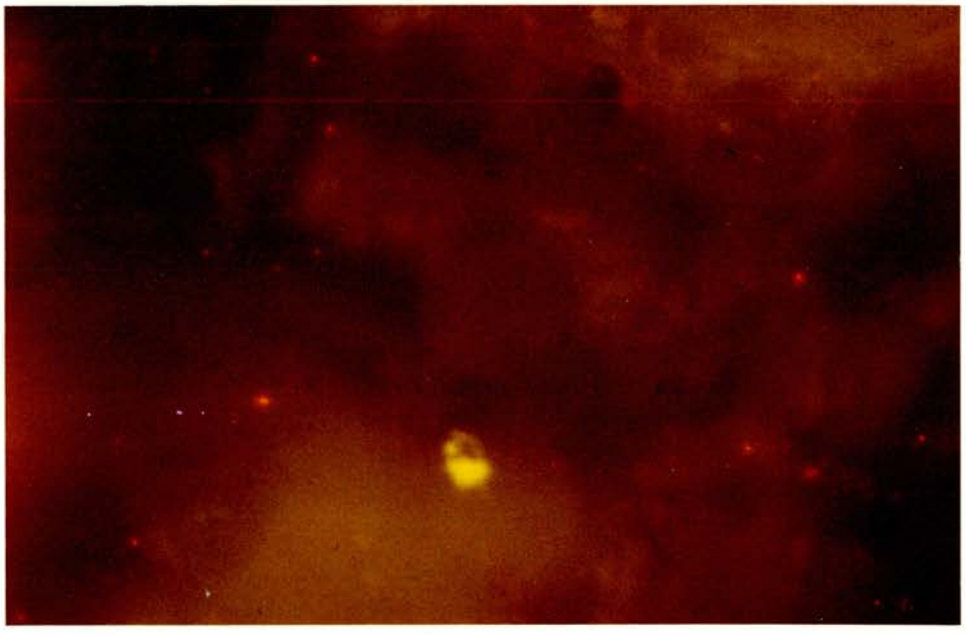
FIG. 12.4,b.

Indirect immunofluorescence of horse dermis; antihuman IgE preparation of section from biopsy 1 (Table 12.4). x440

FIG. 12.5.

Toluidine Blue (pH 4.2) overstaining of the anti human IgE fluorescent section shown in Figures 12.4a and b. Arrow indicates the metachomatically stained cell corresponding to the fluorescent cell in Figure 12.4b. x440





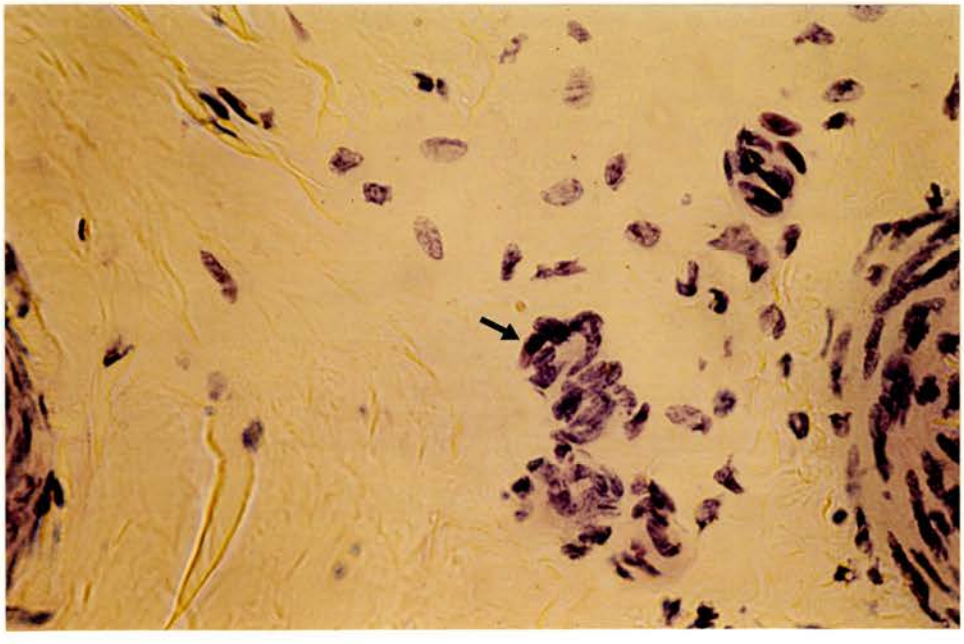
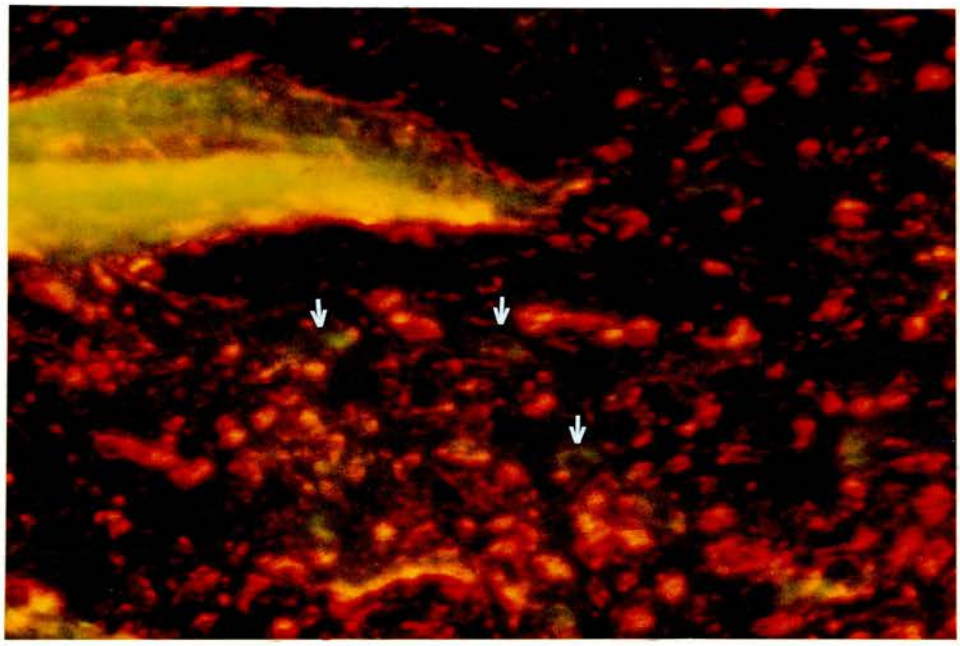


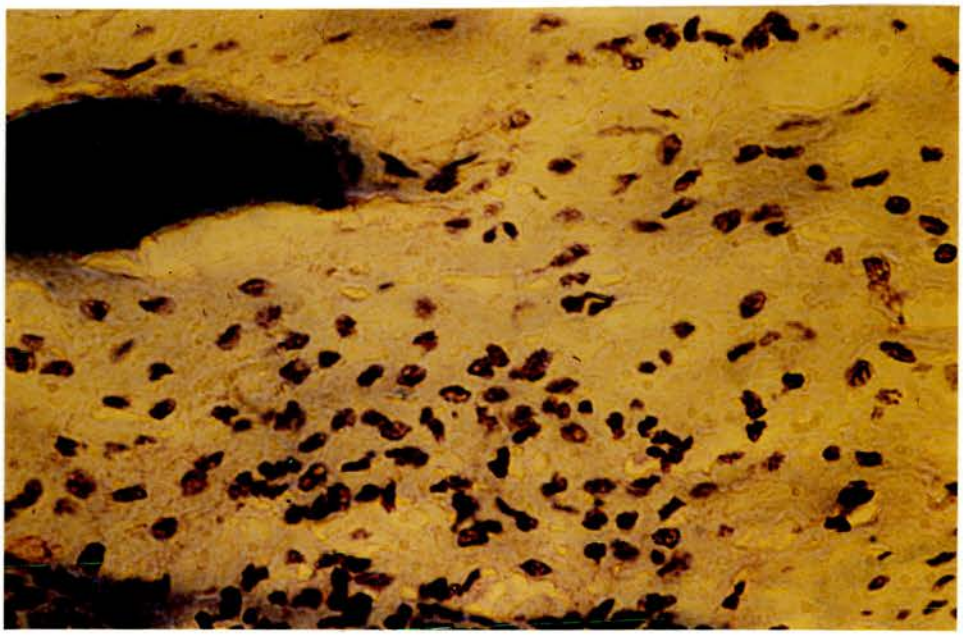
FIG. 12.6.

Indirect immunofluorescence of horse duodenum; anti-human IgE preparation showing cellular cytoplasmic fluorescence within the lamina propria. (arrowed) x440

FIG. 12.7.

Toluidine Blue (pH 4.2) overstaining of the fluorescent preparation shown in Figure 12.6. x440





from the three duodenal preparations.

12.4.4. DISCUSSION

The results of this study are inconclusive although encouraging in that they present some evidence of binding of sheep-anti human IgE globulin to cells within horse dermis and duodenal lamina propria. The identity of these cells was not conclusively established. However the cytoplasmic fluorescence encountered is typical of FITC conjugated anti-IgE staining mast cells in other species (Tada and Ishizaka, 1970; Halliwell, 1973; Mayrhofer, et al., 1976). Although IgE is thought to exert its biological activity on the mast cell membrane Simson et al., (1977) have shown that homo- and heterocytotropic antibodies may bind to the mast cell granule matrix.

A number of technical difficulties were encountered during the experimental procedure, principally the failure of toluidine blue overstaining of mast cells within the fluorescent sections. Although this problem was not encountered by either Halliwell (1973) or Healey and Gaafar (1977) in their studies of canine mast cells, it was reported by Mayrhofer et al., (1976) in their studies of rat mast cells and plasma cells. The problem may possibly be overcome by the use of alternative counterstains, particularly the copper thalocyanine dyes such as Alcian Blue. However further work is necessary to establish this.

Tissue loss during counterstaining was encountered by Halliwell (1973) who resolved the problem by using adjacent sections for determining the source of cellular IgE fluorescence. However in the present study the individual cells within adjacent sections could not be associated with cells in the test sections, though in thinner sections this may be possible.

EQUINE HOMOCYTOTROPIC ANTIBODY: GENERAL DISCUSSION AND CONCLUSIONS.

The results of this preliminary study have identified a homocytotropic antibody in horse and pony serum. Although it will be necessary to isolate the antibody before definitively identifying it as a homologue of human IgE, the limited data on the functional, physicochemical and immunological characteristics of this antibody all satisfy Vaerman's (1970) 3 orders of criteria for interspecies protein homology.

These 3 criteria are;

(i) Immunological cross reactivity and amino acid sequence homologies:

Both Vaerman, Heremans and Von Kerchoven (1969) and Neoh, et al. (1973) have stated that immunological cross reactivity is a practical alternative to sophisticated amino acid sequencing as a means of demonstrating homology in primary polypeptide chain structure. The results of the present study indicate some degree of immunological cross reactivity between human IgE and horse homocytotropic antibody, thus satisfying this first order criteria.

Antigenic cross reaction of these two heterologous proteins is not surprising in view of the cross reactivity of human IgE with the reaginic antibodies in other mammalian species. Furthermore antigenic cross reactivity of the heavy chains of human and horse immunoglobulins G, M and A has been demonstrated (Allen, Berger and Helms, 1968; Vaerman et al., 1971;

Esteves and Binaghi, 1972; McGuire and Crawford, 1972; Neoh et al., 1973).

However, previous studies of antigenic cross reactivity of human IgE and presumed horse IgE have found scant evidence of cross reactivity. Neoh et al., (1973) failed to demonstrate human-horse IgE interaction by radial immunodiffusion using a single horse serum and radiolabelled avian and mammalian anti-IgE globulin. However, this technique may be, by these authors' own admission, insufficiently sensitive to detect low levels of the immunoglobulin in the test serum. In the second report, Nielsen (1977) based his quantitative study of heterologous IgE antigenic cross reactivity on both radial immunodiffusion and the ability of the test reagin to bind to rat peritoneal mast cells prior to labelling with radiolabelled anti-human IgE. The results were expressed as the percentage of the total radioactivity remaining in the washed immunodiffusion gel and the washed rat mast cell deposit after the heterologous serum-radiolabelled anti-human IgE interaction. The results obtained by both techniques showed remarkable agreement in each species examined, which included the rat. This suggests that the IgE homologues in each species had an equal affinity for the receptors on the rat mast cell membranes and also that this affinity is the same as that of rat IgE. This is an unusual finding in the light of the work on

heterologous IgE - rat mast cell binding which has demonstrated marked instability of this interaction in contrast to the rat reagin-rat mast cell interaction (Plaut, et al., 1973; Wyczolkowska and Provoust-Danon, 1976). However, assuming some degree of interspecies reagin cross reactivity, a major experimental variable in determining the extent of interspecies antigenic homology using Neilsen's method, which employs excess anti-human IgE, will be variation in reagin levels in the test serum. This factor appears not to have been considered by this author. Nevertheless, Neilsen did show some 3% cross reaction of human IgE and a horse homologue. Although this was less than in other species, such as the cow, sheep and pig, it may be significant in view of the work of Esteves and Binaghi (1972) who readily demonstrated antigenic cross reactivity between human, sheep, pig and cow IgG but were able to demonstrate human-horse IgG homology only using anti-human IgG sera produced by prolonged immunization of experimental rabbits.

(ii) Association of a protein with a particular function:

The midge antigen specific homocytotropic antibody in horse serum appears to be involved in the immunopathogenesis of R.S.D.. The pathological and epidemiological features of this disease have indicated that it is an acquired hypersensitivity to biting midges,

probably C. pulicaris (Mellor and McCaig, 1974; Baker Quinn, 1978). In man, IgE mediated hypersensitivity is involved in anaphylactic reactions to insect venom (Sobotka, et al., 1974). Thus the homocytotropic antibody in the horse satisfies this second criterion of homology with human IgE.

(iii) Physicochemical and biological homology:

The thermolability and mercaptoethanol sensitivity of equine homocytotropic antibody is typical of the reagins in man and other species. The prolonged persistance of the antibody in homologous skin is also typical of the reagins of other species. On DEAE cellulose in Tris buffer (pH 8.0) the elution pattern of the homocytotropic antibody from whole horse serum corresponds to that of IgE from human serum. These observations satisfy to some extent this third criterion of interspecies protein homology. However, these observations cannot exlude the possibility of homocytotropic activity being associated with IgA in the horse. Similarly, Barratt (1972) and Halliwell et al., (1972) identified IgE homologues in the pig and dog but were unable to exclude the possibility of skin sensitizing antibodies of the IgA class occurring in these species. Further work will be necessary before the homocytotropic activity in horse serum can be attributed exclusively to an IgE-like antibody.

The local anaphylactic responses in horse skin

observed in this study is biphasic and is similar in many respects to the late cutaneous anaphylactic response in man. Whether the horse is capable of the transient wheal and flare type response is not known, and further work is necessary to establish the specific mechanisms involved in the delayed phase of the response. This is of importance in that recent reports have described prolonged responses following direct I/D testing of C.O.P.D. affected horses with fungal antigens, which the authors have suggested represent Arthus-like reactions (McPherson et al., 1979a; Halliwell, et al., 1979). These responses may be wholly or in part prolonged homocytotropic antibody mediated reactions, and an understanding of the humoral immunopathogenesis of C.O.P.D. will depend upon assessing the respective roles of Type I and Type III hypersensitivity reactions.

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APPENDICES.

APPENDIX I.

SERUM ALBUMIN CONCENTRATION (g/L) OF 20 HEALTHY HORSES DETERMINED USING TWO INDEPENDANT METHODS:

(a) Biochemical Assay (BCG Binding; Doumas *et al.*, 1971).

(b) Electrophoresis.

SERUM	(a) BCG Binding	(b) Electrophoresis
1	33.1	33.6
2	38.1	32.8
3	33.8	30.2
4	33.1	32.5
5	35.2	33.7
6	33.8	30.5
7	40.3	35.5
8	29.1	29.1
9	30.5	37.8
10	33.1	32.9
11	28.0	30.6
12	26.7	28.2
13	26.4	26.7
14	28.4	32.2
15	35.8	33.3
16	32.3	30.2
17	32.1	27.7
18	39.1	31.6
19	29.4	29.2
20	28.4	29.5

APPENDIX 2

TOTAL SERUM AND ELECTROPHORETIC ZONE PROTEIN CONCENTRATION (g/L) IN THE SERA OF CONTROL HORSES AND PONIES:

(A) Healthy Horse Population (3.2.2.)

(B) Healthy Pony Population (3.2.2.)

FOR ANALYSIS, DETAILS OF SEX AND AGE ARE INDICATED IN A.

(A) HORSES (n = 30).

Animal	Total Protein	Albumin	Alpha-1	Alpha-2	Beta-1	Beta-2	Gamma-1	Gamma-2	Age (years)	Sex *
1	66.9	25.4	5.6	7.2	11.8	5.0	2.9	9.0	9	G
2	62.2	26.8	5.5	5.9	8.0	4.9	1.7	9.1	10	G
3	67.2	25.9	5.4	7.5	12.0	7.1	3.0	6.3	7	M
4	73.4	27.6	6.2	8.1	14.5	6.5	2.4	7.8	7	M
5	73.1	25.1	5.3	8.8	14.5	8.3	1.1	10.0	9	M
6	68.6	25.1	5.6	8.0	11.9	5.5	3.2	9.2	8	M
7	62.7	28.0	6.1	6.1	8.3	3.5	2.6	8.0	2	G
8	71.5	33.6	6.4	7.9	10.7	5.4	1.4	6.1	4	G
9	59.6	32.8	5.1	3.9	6.6	3.9	2.1	5.4	8	G
10	62.3	30.2	7.2	6.9	7.2	3.7	1.6	5.3	13	G
11	65.0	32.5	6.5	6.8	8.1	3.9	1.0	6.2	4	G
12	76.6	33.7	5.4	11.1	12.6	5.4	2.3	6.1	4	S
13	63.5	30.5	6.4	7.9	5.4	6.0	1.6	5.7	4	G
14	65.7	35.5	7.6	4.6	4.3	4.6	2.0	7.2	10	S

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APPENDIX 2 (CONTINUED)

Animal	Total Protein	Albumin	Alpha-1	Alpha-2	Beta-1	Beta-2	Gamma-1	Gamma-2	Age (years)	Sex *
15	57.2	29.1	5.2	5.7	6.6	3.7	2.6	5.7	21	G
16	61.5	37.8	3.7	3.4	6.2	2.8	1.9	6.2	10	G
17	55.1	30.0	4.7	3.6	6.9	3.7	1.9	4.7	8	G
18	59.3	32.9	6.2	4.2	5.0	4.7	1.8	4.4	6	G
19	72.1	30.6	7.5	6.9	9.4	6.9	4.3	7.2	19	G
20	59.4	28.2	7.1	5.1	6.8	4.5	2.1	5.6	9	G
21	72.1	26.7	6.9	8.6	10.5	10.1	2.9	7.2	4	G
22	67.9	32.2	8.8	7.5	5.1	6.8	3.0	4.4	4	G
23	58.3	23.3	8.2	5.3	9.9	4.7	1.8	5.3	8	M
24	68.6	33.3	6.5	7.5	9.3	4.8	2.1	5.2	12	M
25	61.6	30.2	6.8	5.9	5.5	5.9	1.9	5.5	9	M
26	58.4	27.7	5.8	5.6	6.4	5.0	2.0	5.8	7	M
27	60.2	31.6	6.0	5.4	5.1	5.4	1.8	4.8	11	M
28	83.9	31.8	8.8	9.2	15.5	8.0	3.4	7.1	8	M
29	72.9	29.2	8.0	8.8	13.9	6.2	2.2	4.7	6	G
30	68.6	29.5	7.5	8.2	12.3	4.8	2.1	4.8	22	G

* M - Mare; G - Gelding; S - Stallion.

(B) PONIES (n = 47)

Animal	Total Protein	Albumin	Alpha-1	Alpha-2	Beta-1	Beta-2	Gamma-1	Gamma-2
1	76.0	31.9	4.9	9.5	10.6	7.6	2.3	9.9
2	72.6	25.4	7.3	12.0	11.3	8.7	2.2	5.8
3	78.1	23.3	5.6	14.2	18.6	8.7	1.6	6.7
4	86.0	27.1	6.9	16.3	15.0	11.2	2.1	7.3
5	73.2	26.7	4.8	9.5	9.9	12.4	1.8	8.1
6	83.8	33.4	5.5	12.6	15.5	8.4	2.5	7.5
7	97.3	21.4	6.8	21.4	19.4	12.2	3.4	12.6
8	76.5	28.7	7.3	8.4	13.0	9.2	2.7	7.3
9	70.1	25.9	4.6	9.1	13.0	9.5	1.8	6.3
10	84.1	31.1	5.9	10.9	15.1	9.3	3.4	8.4
11	78.3	27.8	4.7	14.5	13.3	7.0	2.7	8.2
12	74.7	27.3	6.0	10.8	14.6	7.8	1.9	6.3
13	73.2	33.3	5.5	9.5	12.8	7.0	0.7	4.4
14	51.5	18.5	3.6	7.2	10.6	5.4	1.3	4.9
15	75.2	29.7	6.8	10.9	12.8	8.3	2.3	6.0
16	70.9	22.6	5.3	15.2	13.8	8.2	2.1	3.5
17	69.4	25.6	5.9	10.4	13.2	6.9	2.4	4.9
18	69.6	28.8	4.9	9.7	12.5	6.3	1.4	5.9
19	73.9	23.3	7.0	12.6	15.5	7.4	3.0	5.9
20	65.6	28.5	3.9	8.2	12.1	6.2	1.3	5.2
21	75.4	27.9	4.5	12.8	14.7	8.7	1.9	4.9
22	57.6	25.9	4.6	6.3	11.5	4.6	0.9	3.7

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APPENDIX 2 (CONTINUED)

(B) PONIES (n = 47)

Animal	Total Protein	Albumin	Alpha-1	Alpha-2	Beta-1	Beta-2	Gamma-1	Gamma-2
23	74.8	27.3	4.9	16.0	14.6	6.7	1.1	4.9
24	64.0	28.1	3.5	6.3	8.7	8.9	1.5	7.0
25	69.3	20.7	4.3	6.7	14.3	12.1	2.0	9.2
26	67.4	26.0	5.2	10.3	10.0	7.4	3.1	9.0
27	68.4	20.0	3.9	9.0	15.0	8.1	2.1	10.3
28	71.5	25.1	5.3	8.0	10.1	11.5	3.0	9.1
29	66.2	20.5	4.8	8.5	13.2	9.3	2.6	7.2
30	62.9	23.5	5.3	6.6	8.7	8.7	1.8	8.1
21	61.6	20.1	3.9	8.7	12.5	6.3	1.7	7.7
32	63.7	22.8	4.5	7.4	15.9	4.5	2.3	6.2
33	66.7	18.9	4.3	8.5	13.2	7.9	2.1	11.9
34	73.7	21.2	4.9	10.1	17.0	7.9	3.2	9.2
35	70.2	19.7	5.0	7.2	16.4	4.8	3.8	13.1
36	61.3	19.9	3.4	6.9	9.7	8.7	1.5	10.9
37	62.2	23.8	4.5	6.3	10.5	7.8	2.0	7.2
38	63.8	22.7	6.0	9.3	11.7	6.6	2.2	6.0
39	63.0	25.2	5.6	7.1	9.0	6.3	2.7	7.1
40	67.5	24.3	4.8	5.8	11.8	9.1	3.5	8.1
41	65.0	23.0	4.9	7.9	12.8	5.9	2.1	8.4
42	71.2	25.1	5.7	7.4	10.8	7.4	3.0	10.2
43	62.1	26.1	4.7	8.3	8.3	4.8	2.2	6.8
44	71.9	23.6	5.6	7.8	14.2	9.8	1.9	9.0
45	68.9	25.8	6.8	10.7	5.5	7.3	1.8	11.0
46	66.0	23.4	5.9	7.8	11.8	4.9	2.1	10.0
47	65.2	26.3	6.1	7.5	9.9	5.2	2.2	8.1

APPENDIX 3

TOTAL SERUM AND ELECTROPHORETIC ZONE PROTEIN CONCENTRATION (g/L) IN THE SERA OF DISEASED HORSES AND PONIES:

(A) C.O.P.D. AFFECTED HORSE POPULATION (4.2.1).
(B) C.O.P.D. AFFECTED PONY POPULATION (4.2.1).

(A) C.O.P.D. AFFECTED HORSES (n = 15).

Animal	Total Protein	Albumin	Alpha-1	Alpha-2	Beta-1	Beta-2	Gamma-1	Gamma-2
1	62.9	26.7	6.0	5.7	6.9	7.6	2.8	7.2
2	62.7	28.2	6.6	5.6	8.5	4.7	2.2	6.9
3	55.6	26.1	5.8	6.1	7.5	3.6	1.7	4.7
4	66.2	24.5	6.3	11.2	9.6	5.0	1.7	7.0
5	66.2	30.1	7.3	8.0	6.6	6.9	1.3	6.6
6	74.0	31.5	7.4	7.8	8.1	10.0	2.6	5.9
7	57.1	32.8	6.9	4.0	6.3	2.6	1.4	3.3
8	64.4	30.6	5.8	7.4	5.8	6.1	1.9	6.8
9	72.0	33.5	6.5	8.6	9.0	6.5	2.2	5.8
10	72.8	33.5	7.3	8.0	7.7	7.7	2.5	6.2
11	73.2	33.3	5.9	7.6	9.9	7.3	1.9	7.3
12	72.1	31.4	6.2	6.9	10.9	6.5	2.6	7.9
13	75.4	24.8	7.9	11.5	11.9	5.7	3.4	10.2
14	69.1	19.0	6.9	11.0	9.3	6.5	3.4	13.1
15	75.4	26.4	7.3	9.3	8.8	9.6	2.3	12.8

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(B) C.O.P.D. AFFECTED PONIES (n = 15).

Animal	Total Protein	Albumin	Alpha-1	Alpha-2	Beta-1	Beta-2	Gamma-1	Gamma-2
1	69.4	34.4	6.6	7.6	5.2	6.9	2.8	5.9
2	74.3	37.1	7.4	7.1	7.1	6.3	3.0	6.3
3	71.2	26.3	6.4	7.1	10.3	11.0	0.7	9.3
4	83.0	28.6	5.4	12.4	12.0	10.8	2.1	11.6
5	68.8	29.6	7.6	6.9	7.9	8.6	1.7	6.5
6	67.3	28.9	7.4	6.7	7.8	8.4	1.6	6.4
7	81.1	33.7	4.9	9.7	12.5	10.0	2.4	7.7
8	69.4	30.9	4.2	8.0	9.4	6.9	2.1	8.0
9	72.2	30.5	4.3	7.9	9.7	7.2	2.9	9.4
10	73.5	17.5	8.3	13.3	11.1	8.1	3.0	12.4
11	56.7	17.6	5.7	9.67	9.1	5.8	2.2	6.4
12	66.2	24.3	7.2	8.9	7.1	8.7	2.3	7.6
13	76.9	27.8	7.3	12.5	11.2	5.6	2.9	9.5
14	82.8	24.6	6.7	12.6	14.5	10.8	1.9	11.8
15	64.3	26.4	5.7	8.0	6.3	6.9	2.3	8.6

APPENDIX 4

EXPERIMENTAL VARIATION IN ESTIMATION OF SERUM TRYPSIN INHIBITORY CAPACITY (STIC).

(a) STIC (mg/ml) of 8 sera derived;

(i) Using a standard trypsin hydrolysis rate curve.

(ii) Using the mathematical expression of Eriksson (1965).

SERA	(i)	(ii)
1	1.45	1.50
2	0.95	0.91
3	1.70	1.64
4	1.45	1.43
5	1.05	1.07
6	1.35	1.37
7	0.85	0.82
8	1.55	1.57

(b) STIC (mg/ml) derived from duplicate assays of 8 sera.

SERA	1st Assay	2nd Assay
1	1.50	1.70
2	1.30	1.60
3	1.90	1.90
4	2.00	2.25
5	1.80	1.60
6	1.20	1.10
7	1.50	1.75
8	2.50	2.20

APPENDIX 4 (CONTINUED)

(c) STIC (mg/ml) derived from 8 repeat assays on a single serum carried out on different days.

ASSAY NUMBER	STIC
1	1.65
2	1.60
3	1.80
4	1.85
5	1.45
6	1.65
7	1.90
8	1.50

APPENDIX 5

Pr PHENOTYPES AND STIC (mg/ml) WITHIN A HEALTHY THOROUGHBRED POPULATION:

FOR ANALYSIS THE INDIVIDUAL ANIMALS ARE SEGGREGATED INTO STALLIONS/GELDINGS, MARES AND FOALS (< 12 MONTHS OLD).

ANIMAL	Pr PHENOTYPE	STALLION/GELDING	MARE	FOAL	STIC
1	LU	+			1.20
2	SU		+		1.10
3	UU			+	1.30
4	FL		+		1.55
5	LL		+		1.40
6	LL			+	2.20
7	LL		+		2.05
8	LU			+	1.30
9	FL		+		0.85
10	FU			+	1.50
11	LS	+			1.10
12	LL		+		2.00
13	LS			+	1.20
14	LS		+		1.40
15	LL			+	1.55
16	FS			+	1.75

CONTINUED/...

APPENDIX 5 (CONTINUED)

ANIMAL	Pr PHENOTYPE	STALLION/GELDING	MARE	FOAL	STIC
17	LS		+		1.30
18	LS			+	0.90
19	LS	+			0.95
20	LL		+		1.05
21	LS			+	1.05
22	LL		+		1.15
23	LS			+	1.25
24	LS	+			1.30
25	LS		+		1.60
26	LL		+		1.55
27	SU		+		0.90
28	SS		+		0.65
29	FL		+		1.35
30	LL			+	1.00
31	LL	+			1.35
32	LL		+		1.70
33	LL	+			1.60
34	LL	+			2.25
35	LL		+		2.15
36	LL	+			1.45
37	LL			+	1.30
38	LS		+		2.20
39	IL		+		1.50
40	IL			+	0.75

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APPENDIX 5 (CONTINUED)

ANIMAL	Pr PHENOTYPE	STALLION/GELDING	MARE	FOAL	STIC
41	LL	+			1.90
42	IL	+			1.30
43	LU		+		2.10
44	LU			+	1.90
45	LU	+			0.80
46	LU	+			1.00

APPENDIX 6

Pr PHENOTYPE, RELATIVE SERUM Pr CONCENTRATION (Pr%) AND STIC (mg/ml) WITHIN A C.O.P.D. AFFECTED POPULATION. FOR ANALYSIS, THE THOROUGHBRED MEMBERS OF THE POPULATION ARE INDICATED.

Animal	Thoroughbred	Pr Phenotype	Pr%	STIC
1	+	FS	99	1.05
2	+	NS	104	2.45
3	+	LU	116	1.70
4	+	NS	75	1.10
5	+	FU	85	0.75
6	+	FL	96	0.90
7	+	SS	87	1.05
8	+	IN	108	2.25
9	+	LN	99	1.20
10	+	IS	114	1.90
11	+	IL	104	1.40
12	+	FL	198	2.25
13	+	LL	121	1.60
14	+	LL	141	1.40
15	-	FS	100	1.00
16	-	FL	144	1.80
17	-	LL	77	1.00
18	+	LU	109	0.85
19	-	LL	90	0.90
20	-	IL	114	1.85
21	-	LS	121	1.60
22	-	IS	71	1.05
23	-	LS	124	1.75
24	-	LW	83	1.30
25	-	LL	99	0.95
26	-	FL	88	0.90
27	-	LU	99	1.00
28	-	IS	104	1.10
29	-	IS	69	1.05
30	-	SU	87	1.60
31	-	FW		
32	-	LN		
33	-	FU		
34	+	LS		
35	-	FW		
36	-	UU		
37	-	LU		
38	-	LL		
39	-	UU		
40	+	FU		

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APPENDIX 6 (CONTINUED)

Animal	Thoroughbred	Pr Phenotype	Pr%	STIC
41	-	WW		
42	-	FU		
43	+	SS		
44	-	NW		
45	-	LW		
46	-	WW		
47	-	LW		
48	-	SW		
49	+	NU		
50	-	UU		
51	+	LU		
52	-	LU		
53	-	FU		
54	-	LU		
55	-	WW		
56	-	LW		
57	-	LW		
58	-	WW		
59	-	LS		
60	-	LS		
61	-	LU		
62	-	NU		
63	-	UU		
64	-	WW		

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Isoelectric focusing of horse acidic prealbumins on thin-layer polyacrylamide gels

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Summary

The paper describes a technique of thin-layer polyacrylamide gel isolectric focusing of horse serum, within a pH range of 4.0 – 6.0, which permits the improved resolution of the acidic prealbumin protein bands. The increased heterogeneity of the Pr prealbumin antiprotease allele products apparent using this technique is described and discussed in detail, and the potential use of the technique in routine Pr phenotyping is considered.

Introduction

On acidic starch gel electrophoresis of equine serum, a large number of prealbumin protein bands become apparent (Braend, 1967). The more anodal and cathodal bands are grouped into two genetically determined polymorphic protein systems, Pr and Xk (Braend, 1970). Between the Pr and Xk systems lie, in order of increasing electrophoretic mobility, a discrete group of bands designated Xh (Braend, 1967) and two antigenically and functionally distinct proteins, corresponding to the Xc/Xd group of bands suggested by Braend (1967) (Matthews, 1979). The Pr protein has been identified as the homologue of human alpha-1 antitrypsin and the 'Xc' protein as an alpha-1 protease-inhibitory protein (Matthews, 1979).

Difficulties encountered in the technique of acidic starch gels in relation to Pr phenotyping were mentioned by Scott (1976) and are reflected in the results of both the 1975 and 1977 horse blood typing comparison tests (Buis, 1976; Storset & Braend, personal communication). In man, isoelectric focusing on thin-layer polyacrylamide within a limited acidic pH range has superseded acidic starch gel electrophoresis in the study of genetic variants of the acidic prealbumin, alpha-1 antitrypsin (Allen et al., 1974). For these reasons, isoelectric focusing was used to

study the multiple molecular forms of the acidic prealbumins as part of an investigation into the possible role of serum antiproteases in the onset of chronic obstructive pulmonary disease in the horse. This paper details the isoelectric focusing technique used and describes and discusses the resulting isoelectric heterogeneity of the Pr protein.

Materials and methods

Serum samples were obtained from 33 Thoroughbred sire-dam-offspring groups and a number of horses and ponies of undetermined breeding. Serum was stored at –20 °C for up to two years until used. All Thoroughbred serum samples had been Pr phenotyped by a modification of the discontinuous acidic starch gel electrophoretic technique described by Fagherol (1972). This modification involved the adjustment of the relative concentrations of the gel buffer stock solutions to give a final pH of 4.3 – 4.5. Thirty sera from the population of animals of undetermined breeding were Pr phenotyped by the horse blood typing laboratory in Oslo according to the technique of Braend (1970).

Thin-layer polyacrylamide gel electrofocusing in the pH range 4.0 – 6.0 was carried out using the LKB 2117 Multiphor system.

The gel frames (250 mm × 125 mm) were made up of a thick glass plate (LKB 2117-105), a thin glass plate (LKB 2117-104), a rubber gasket (LKB 93 90 6010) and a second thick glass plate held together with bulldog clips. The gel contact surfaces were thoroughly cleaned with detergent, dried and wiped with ethanol before use.

Three gel stock solutions were used:
– *acrylamide*: 58.1 g acrylamide was dissolved in 200 ml distilled water, passed through a 0.22 μm-pore size filter and stored at 4 °C;
– *NN'-methylenebisacrylamide*: 1.8 g bisacrylamide was dissolved in 200 ml distilled water, passed through a 0.22 μm-pore size filter and stored at 4 °C;
– *riboflavine*: a saturated solution in distilled water was prepared and stored at 4 °C.

The gel was prepared by mixing 13 ml acrylamide and 13 ml bisacrylamide with 30 ml of 25 % (w/v) sucrose solution, to which 3.5 ml ampholine, pH 4.0 – 6.0 (LKB 1809-116), withdrawn under sterile conditions, was added. To this mixture 0.4 ml riboflavine solution was added as a polymerising agent.

The mixture was degassed by strong suction into a 50 ml plastic syringe through a 19-gauge needle, the needle was removed and the mixture poured into the frame. Care was taken to exclude air from the frame during pouring.

The gel was allowed to polymerise at room temperature under ultraviolet light. Polymerisation was usually completed within 2-3 hours, indicated by a change in refractive index at the periphery of the gel. The gels may be stored overnight at 4 °C with no effect on their subsequent use.

After careful removal of the two thick glass plates, serum samples (approximately 5 μl) on rectangular strips of LKB inserts (2117-106) were applied along the length of the gel, 1 cm from the cathodal wick, using a paper template as a guide. The anodal and cathodal wicks were applied to the gel after soaking in 1 *M* phosphoric acid and 1 *M* sodium hydroxide respectively, giving an electrode distance of 100 mm.

The gel on the thin glass plate was placed upon a layer of water laid over the cooling plate of the Multiphor. The focusing lid allowing voltage application across the breadth of the tank was fitted, ensuring good electrode contact with the wicks on the gel surface. After switching on the cooling system, the power pack was set to deliver a maximum of 1000 V and 30 W. The initial potential difference of 400 V increased to maximum during the run and the initial current of 30 mA fell as the pH gradient formed. Electrofocusing was completed after a 6-hour run.

The gels were stained for 30 minutes at 60 °C with Coumassie Brilliant Blue (Vesterberg, 1972). Destaining takes place overnight using an 8:3:1 water/ethanol/acetic acid solution. The gels may be mounted for prolonged storage in a glycerine/destaining solution (1:4) mixture.

The stained protein bands on the gels are best visualised with transmitted light.

Results

Thin-layer polyacrylamide gel electrofocusing of equine serum in the pH range 4.0 – 6.0 is a highly reproducible technique, permitting the resolution of a large number of acidic protein components of differing isoelectric points (pI). The separation of these components into distinct groups within the lower range of the pH gradient appears essentially similar to that of the prealbumin proteins after acidic starch gel electrophoresis (Fig. 2). The Pr and Xk proteins may be identified by their polymorphic nature. Between the Pr and Xk proteins, a number of bands may be appreciated. These appear distributed into three discrete groups (Fig. 2) similar to the distribution on acidic starch gels (Braend, 1967).

A 6-hour electrofocusing time resulted in optimal resolution of the proteins. By this technique, a 2-hour period as recommended for the electrofocusing of human alpha-1 antitrypsin variants resulted in incomplete isoelectric separation which was not comparable with acidic starch gel electrophoresis.

The Pr protein

With an effectively linear pH gradient, the pI of the Pr protein will lie approximately between pH 4.1 and 4.5.

On acidic starch gel electrophoresis, the Pr phenotypes are determined by a minimum of 10 codominant alleles, designated *F*, *G*, *I*, *L*, *N*, *S*, *T*, *U*, *W* and *Z* in order of decreasing electrophoretic mobility (Braend, 1970; Scott, 1976, 1977). By this electrofocusing technique, the appearance of the Pr phenotypes (Fig. 1) is

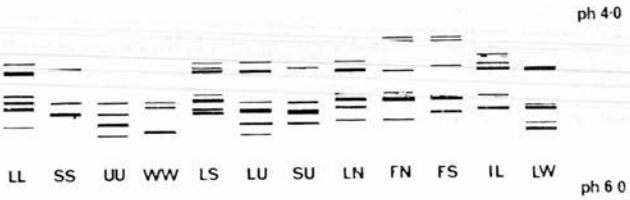

Fig. 1. Schematic appearance of some Pr phenotypes after isoelectric focusing within the pH range 4.0 - 6.0.

basically similar to that on starch gels. However, the number and resolution of the bands in the Pr system is increased, each allele controlling a multiple band pattern of one or two intensely stained bands, with a variable number of lightly stained bands.

After isoelectric focusing, the PrF allele products, recognized only in heterozygotes in this series of animals, appear as indistinct double bands near the lower pI limit of the protein, with a third band of pI indistinguishable from the major PrN allele product. These bands are shown in the FN (Fig. 2 Nos 9 and 10), FL (Fig. 3 No 5) and FS (Fig. 3 No 9) phenotypes. The PrI allele products, again recognized only in heterozygotes in this series, appear as two zones with pI distributed about that of the most acidic PrL allele products, with an additional band of pI slightly more acidic than the common PrN and PrF allele product. These bands are shown in the IL phenotype (Fig. 3 Nos 12 and 21). In cases where the PrI allele products are weakly expressed, the two more acidic zones may be difficult to visualise.

The PrL allele product shows the three bands characteristic of the homozygote on acidic starch gel electrophoresis (Braend, 1970). However, two additional bands of intermediate pI are apparent in the homozygote (Fig. 2 Nos 1 and 2), the pI of the least acidic of these two bands being indistinguishable from the apparently common PrN and PrF bands. In a number of PrL homozygotes, one or two additional bands are apparent, of lower pI than those already described (Fig. 2 Nos 15, 16 and 17). The more intensely staining of the more acidic PrL bands may, in some animals, be separated into two bands of close pI, as shown in an LL type in Fig. 3 No 3. The less acidic of these two 'sub' bands has a pI close to a band, considered to be a PrS allele product, shown in the adjacent LS phenotype (Fig. 3 No 2) and in an SS phenotype (Fig. 2 No 4). An LS type with only the more acidic of these 'sub' bands is shown in Fig. 3 No 13.

The PrS allele product appears heterogeneous. In addition to the acidic band already described in Fig. 3 No 2, the allele product appears to have a major band of varying pI. In some animals, the pI of this band is similar to that of the least acidic of the major PrL bands as seen in the SS type (Fig. 2 Nos 4 and 5), while in others the pI of the S band is distinctly less acidic pI than the PrL band, as seen in the SU (Fig. 2 Nos 13 and 14) and LS types (Fig. 3 Nos 2, 7, 8, 13 and 17). The

Fig. 2. Isoelectric focusing of horse serum on thin-layer polyacrylamide gels within the pH range 4.0 - 6.0. The presumed limits of the acidic prealbumins are shown and the Pr phenotypes of the individual samples given. A human MZ alpha-1 antitrypsin phenotype is shown for comparison (sample 20).

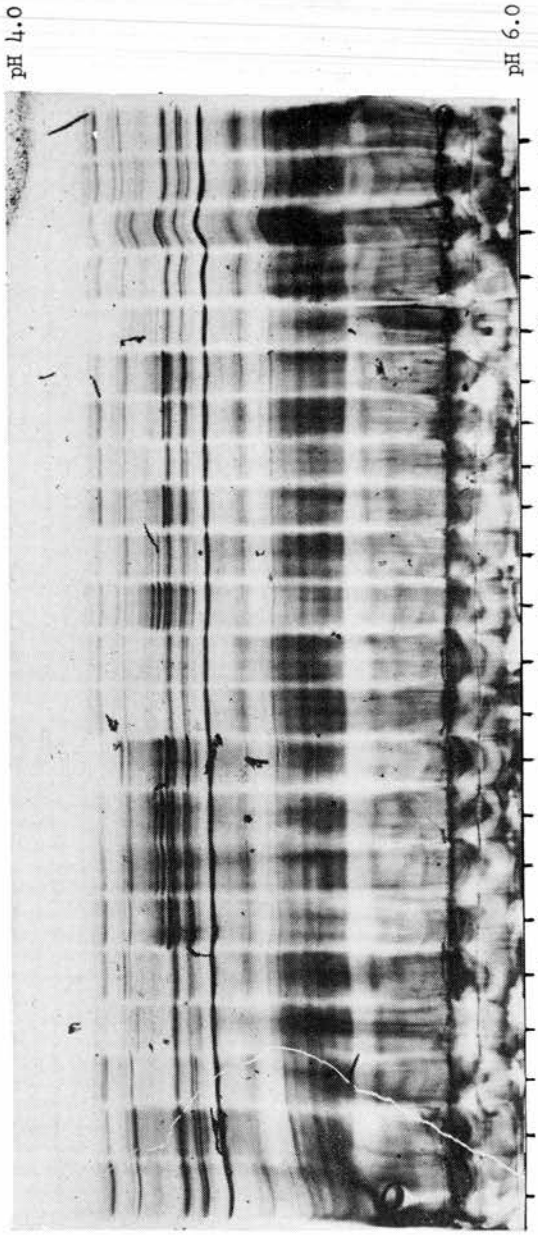

Fig. 3. Isoelectric focusing of horse serum on thin-layer polyacrylamide gels within the pH range 4.0 - 6.0.

PrS product has an additional band of pI similar to the apparently common PrF, PrN and PrL bands.

The PrU allele product (Fig. 2 Nos 7 and 12) appears as two distinct bands, the more acidic band having a pI similar to the major S band. In some cases, two additional bands of more and less acidic pI's respectively are apparent.

The protein bands considered to represent the PrW allele products are shown in Fig. 2 No 3 in a sample taken from a non-Thoroughbred animal.

Discussion

Isoelectric focusing provides a readily reproducible method of examining the Pr phenotypes of relatively large numbers of samples simultaneously. Although the highly polymorphic Pr locus is reported to be the single most effective system in the detection of falsely assigned parentage in the horse (Scott, 1976), only 7 of the 22 laboratories included in the 1977 horse blood comparison test (Storset & Braend, personal communication) reported Pr phenotypes. Amongst those 7 laboratories, however, there was only limited agreement as to the correct identity of the Pr allele products. Similar disagreement on Pr phenotypes was reported by Buis (1976) after the 1975 horse blood comparison test.

In comparison to acidic starch gel electrophoresis, this isoelectric focusing technique results in an increased number of bands in the Pr region. Recently, Ek (1979), using antigen-antibody crossed electrophoresis with monospecific anti-Pr antiserum, has identified a number of additional bands in the Pr system after starch gel electrophoresis. Some of these bands are weak or inapparent after routine glycoprotein staining of acidic starch gel, and some lie cathodally to the Pr bands originally described by Braend (1970). The results of Ek (1979) are compatible with the previously undescribed Pr bands apparent after isoelectric focusing.

Although the PrF allele products are easily identified after starch gel electrophoresis, after isoelectric focusing they are relatively inapparent, possibly due to the pI of the more acidic bands approaching the lower limit of the pH gradient. However, extension of the pH gradient towards pH 3.5 may correct this anomally.

The bifid appearance of the more acidic of the major PrL bands may represent the genetically determined variation in the L zones alluded to by Braend and Storset (1979). The identification of this variant after isoelectric focusing would permit investigation of its possible genetic control. Due to their variable appearance, the additional bands of lower pI observed in only some PrL homozygotes may belong to a separate protein system or represent Pr isoelectric variants independent of the gene products. The heterogeneity of the PrS allele product may be due to two separate alleles, the more acidic variant of pI similar to the L band being the PrT allele product as described by Braend (1970).

The protein bands considered to represent the PrW allele product was not observed in any of the Thoroughbred horses in this series. It differs from the allele

product on starch gels, as described by Braend (1970), in that on isoelectric focusing the stronger of the two bands of more acidic pI lies much closer to the major band than on acidic starch gels. It is possible that these bands, here considered to be PrW allele products, may represent a subdivision of the PrU allele product as indicated by Braend & Storset (1979). Further investigation, using this technique, will be necessary to establish this.

Despite the complexities of the Pr phenotypes after isoelectric focusing, resulting from the multiplicity and part identity of the allele products, the technique could provide a standard method for routine Pr phenotyping and an accurate tool in the investigation of new variants at the locus.

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